

Europe runs before it walks

The European Commission threatens dire consequences if it is not given a bigger budget for research. But its case is fragile, at best

THE European Commission seems to be heading for a fruitless confrontation with its sponsors, the twelve governments now members of the European Communities. Although this will not be the first time that the Commission has been at loggerheads with the governments of which it is the titular servant, and although there will be general sympathy with the Commission's frustration at the slow pace of change in Europe, it is a pity that the Commission has picked on the research budget on which to do battle. The difficulty is that the Commission is talking as if it might tell member governments that if it cannot have \$8,000 million (over five years) to spend on its "framework programme" of largely applied research, it will let the European research enterprise go hang (see *Nature* 324, 291; 1986). European researchers will be the chief losers, but the Commission's own European ideals will also take a bashing. The best hope now is that the Commission is merely bluffing. Otherwise, it should think again.

The nature of the row is best understood and is only understandable in terms of the Commission's own constitution. Those who wrote the Treaty of Rome which legitimized the European Economic Community and Euratom (there was previously the Iron, Steel and Coal Community) believed that their ultimate goal of a united Europe would come about only as member governments were given proof that Europe would work in small ways. Ingeniously, the founding fathers legislated for a kind of government-in-waiting (the Commission) whose immediate function would be to formulate policies for approval by member governments. In the short run (which is now), the Commission would propose and member governments would dispose, by the agency of what is called the European Council. But in the long run, the calculation ran, member governments would be so content with the developing idea of Europe that they would cede more and more executive power to the Commission and would use the European Parliament as a common legislative body that would also breathe down the Commission's neck. This dream is still a very long way from fruition. The Commission, understandably, is frustrated at being kept a dogsbody for quarrelsome governments, and this frustration has now spilled over onto its policy for research.

Discontents

If discontent with the snail's pace of change were the only touchstone, the Commission would be surrounded by friends. One prominent companion in discontent, but not necessarily an ally, is President François Mitterrand of France, who has been pressing for the past five years for more technical collaboration within Europe and, for the past eighteen months, for a substantial move towards European integration on a wider front. The Commission now seems to regard President Mitterrand's more recent initiative as a licence for insisting that member governments must at last be forced to collaborate on something, and has picked out research. For the time being, it seems prepared to overlook the more glaring of the defects of its enabling treaty — the assumption that little would happen in foreign affairs where, in the event, members' interests most often coincide and the legal provisions that compel collaboration in agriculture to the

great cost of European consumers and cheaper food producers elsewhere in the world. Two-thirds of Europe's energy in the past 30 years, and a great deal of mutual recrimination among the Communities' members, have been occasioned by the unkeepable promise to 1950s' farmers that they would be protected from the discomforts of clearly impending technical change. The Commission knows (it has tried) that reforming the Common Agricultural Policy would do more to liberate European energies than a coordinated policy on applied research, however well conceived the programme. Yet the Commission is so exercised about the budget for its framework programme that M. Jacques Delors has thrown his reputation behind the campaign for \$8,000 million.

Misunderstanding

The Commission's pitch for a larger research programme would be more telling if its record on the conduct of scientific affairs were more convincing. Unfortunately, its record is poor, to say the best of it. Throughout the long argument on regulations on environmental pollution in the 1970s, the Commission too obdurately clung to the notion that pollution must be controlled by emission standards, so as to be fair to manufacturers of the same kind wherever they might be located, and obscurantistly resistant to the notion that what matters is the degree to which the environment is polluted. On many occasions, it seemed that the difficulty was the Commission's failure to understand the difference. More recently, the Commission has been responsible for two quite shocking assaults on good sense in its handling of essentially technical issues. Thus, in the days after the nuclear accident at Chernobyl in April, the Commission hurriedly organized a meeting to agree on common standards of when it would be prudent to ban radioactively contaminated food and concluded that food should not be sold with more than 1,000 Bq per kg—a standard roughly an order of magnitude more stringent than necessary. Worse still, the Commission has decided to use its powers for the regulation of agricultural produce to ban the use by European farmers of natural growth hormones (bovine growth hormone, perhaps manufactured synthetically, to make more beef, for example). It is generally agreed that such a practice would damage consumers, the people who might eat meat grown in such a way. The Commission's motives have simply been to restrict the embarrassing productivity of European farmers. When the ban was declared (it comes into effect this month) the commissioner responsible for agriculture said that there were occasions when "politics must take precedence over science". Is that the kind of organization to which people will willingly entrust \$8,000 million for research?

The Commission will no doubt complain that these administrative matters have nothing to do with its reputation for the conduct of research, but even that is only half true. In its administration of the Communities' four research centres, the Commission has been over-sensitive to political (especially Italian) objections to the suggestion that some of them might be closed. The Commission's sponsorship of fusion research, on the other hand, is admirable. There is no suggestion that the major programmes in applied science have been tainted by



political considerations, but the most interesting of them, that called ESPRIT (where the last two characters stand for information technology) is too young for its benefits to be properly evaluated and costed. Most probably, the Commission is right to think that there are good works it might perform in fields like this, but little would be lost and much might be gained if it set out to foster collaboration in research with much more deliberation.

This is not to say that the Commission must forever drag its feet on research. Curiously, there are many causes that the Commission has itself in the past espoused that could benefit from encouragement — and a little money. Only three weeks ago, the annual general assembly of the European Science Foundation was wringing its hands over the poverty of its plan to build 'networks' of European scientists, ironically stimulated by a study sponsored by the Commission a few years ago. The Commission could also usefully (as it may yet) listen to appeals such as that from the group of European radioastronomers who seek a modest 16 million European Currency Units (roughly £11.4 million) over five years to establish a centre for the analysis of data gathered during very-long baseline measurements. Such projects are not, of course, alternatives to a programme of applied research, but the fact that they go begging is a sign that the Commission has not yet begun to work effectively on the common ground where success would come most easily and bring the greatest benefits — the effective integration of the technical community without which a common research enterprise will have little purpose. □

Licensing the spooks

The British and US governments are both embarrassed by the doings of their undercover people.

WHO would have known, until the British government's attempt to prevent in the Australian courts the publication of a book on its security service that Lord Rothschild, the embryologist and merchant banker, was once a member of that same service, called MI5? This is nevertheless what has emerged from the embarrassing (for the British government) disclosure that Rothschild acted as the intermediary in bringing together Mr Peter Wright, once also a member of MI5 and now living in Australia, and Mr Chapman Pincher, who published a racy account of Wright's tale three years ago. Few will think the worse of Rothschild now; some will now put him in the same daredevil category as Colonel Oliver North, the staff member of the US National Security Agency dismissed last week for alleged gun-running (to Iran) and now described by President Reagan as a national hero.

Popular heroics apart, there is nevertheless a case for believing that spying and spy-catching have got out of hand. Every few months, there seems to be either a serious international incident or a serious domestic crisis in some major capital because of spooky doings (Auckland Harbour, for example). Then there are retaliations (the name of Daniloff refers). The obvious difficulty is that espionage and counter-espionage are unregulated. The obvious solution is that governments employing secret agents should be required to register all salaried agents' identities with an international authority, which would keep the information secret unless asked by the registrant to disclose it (perhaps secretly) to a third party. That way, it would be possible for governments more easily to arrange swaps for agents or even to find out whether people arrested in foreign capitals were in their employ or were merely innocent visitors. By becoming licensed in this way, secret agents would be able to trade self-esteem for the dare-devilry that now sustains them, and would be less inclined to write their memoirs. So long as the international authority were not itself penetrated by agents, the whole business would be made less serious a threat to international good relations; international spy crises might, for example, be defused by mutual consent. Would not such a scheme be worth a trial? □

Action against AIDS

Governments are moving quickly on publicity about AIDS; should they be doing more?

BOTH the British and the French governments have responded well to the latest alarm about the spread of AIDS (acquired immune deficiency syndrome). In both countries, the governments have decided that public education must take precedence in any serious attempt to break the chain of infection by the virus. Plainly that makes sense. Unless people know which features of their personal behaviour expose them to the risk of infection, their self-interest will not coincide with the needs of programmes of public health aimed at averting what could be a cataclysmic infection. One of the several haunting aspects of the problem is that there remain so many features of the natural history of AIDS that are, as yet, unknown. It is not yet known when and for how long, in the course of an infection, a person is capable of infecting others. Whether all those infected, or only a proportion of them, eventually develop AIDS, is another imponderable for the time being. Little is known about the relative risks of transmission in different sexual acts. The case for acting now is that, if all the answers to such questions were gloomy, large proportions of us or our successors would be killed.

It is a pity that young people, at the beginning of their sexual lives, must be the chief targets of public education, but that, too, makes sense; it is not, as usually the case, that young people are especially deserving of consideration (true though that remains) but that they could be especially effective agents for the transmission of a socially damaging and usually venereal disease. To say the least, a young person, during the remainder of his or her lifetime, will on the average have more sexual partners than an older person and will therefore, on the average, be a potential source of infection for a greater number of people. It is also possible that some young people may be unusually promiscuous. To their credit, both governments appear to have faced this part of the problem that confronts them squarely. The British government, with its reputation for being careful about money, has surprised its taxpayers by deciding to spend twice the sum it first thought of (£20 rather than £10 million) on immediate publicity. The French government is wisely emphasizing the need that people should have a ready opportunity of a test for antibodies.

None of this, unfortunately, will throw light on the present incidence of the infection (as distinct from the disease it may ultimately cause). Those who voluntarily seek an antibody test will often be people who suspect they may have run a particular risk. The routine testing of blood offered at transfusion clinics may similarly, as in the United States, be biased by people's recognition that a blood transfusion is the most convenient way of being tested. That is why some means of random testing would, in present circumstances, be beneficial; it would then be possible to tell objectively how great is the present pool of infection, a necessary starting point in estimating more accurately the rate at which infected people progress to overt disease. Schemes for the anonymous testing of pregnant women at antenatal clinics have similarly been advocated as ways of monitoring the spread of AIDS through heterosexual intercourse. The snag is that, in these enlightened days, it is unethical for physicians to test a person's blood without telling him or her what is afoot, whereupon it would be unethical not to disclose the results. The result is that information that would be invaluable for an assessment of the present problem, and which could be collected easily, is not available. The reasons for this self-denial are admirable, but the outcome is ignorance on an important point. If the AIDS problem becomes more serious, societies will find themselves considering a variety of illiberal measures to protect themselves against the pool of those infected; on the face of things, anonymous testing whose results are not disclosed might be a lesser evil that should be more quickly allowed. □

French universities

Student protests succeed in postponing new law

FOR 1986 read 1968 — the year of revolution in French universities which led finally to the resignation of General de Gaulle as President of France. Last week, more than half a million French students and schoolchildren were on the streets to protest against a new law for the universities which, students claim, will tighten up entry requirements, reverse the right of free entry to universities that was won so bitterly by, in many cases, the demonstrators' parents nearly 20 years earlier. Right-wing Prime Minister Jacques Chirac, who dealt with the events in 1968 as a protégé of de Gaulle's prime minister and later president, Georges Pompidou, this time had no stomach for a fight. He told his minister for the universities and research, Alain Devaquet to withdraw the bill for revision.

Resignations do not seem to be in order this time, however. Chirac is not going to see the fall of himself or his government over the issue when the national budget and the economy is his main theme; and the socialist President, François Mitterrand, who might be contending with Chirac in the presidential elections of 1988 and had no involvement in the preparation of the bill, must be laughing up his sleeve. Furthermore, Devaquet, whose law was a compromise between various pressure groups, is said to have many sympathies with the students, and may see the demonstrations as giving weight to his own position. The losers so far in the battle would appear to be 'les émigrés', that group of university staff which was silent, or powerless, during the previous socialist adminis-

tration but which now that a right-wing government is in power has been calling for much greater power for the university professors in France, both in the selection of students and over the control of research funds. In the latter case this would have entailed the dismemberment of the French national research council, CNRS, a battle the *émigrés* recently lost; now, it seems, their battle for more control over their students, 40 per cent of whom leave after one year in university, has also gone against them.

There is thus a good deal of despondency among the rightist camps in French universities this week — if this government cannot help them none can. On the other hand, French natural scientists, who number few of the *émigrés* among them, are more cheerful. Many deplored yet another attempt to reform the universities, and most thought the new bill's efforts to divide departments into single-subject faculties retrogressive, likely to reduce interdisciplinary research and to recreate the pre-1968 professional fiefdoms.

Another common complaint of researchers about the new bill was that it threw away the fledgling French PhD, just two years old, to put back the old system of multiple theses and doctorates of various levels which was incompatible with research qualifications outside France and reduced international mobility. The bill before parliament last week, which resulted in the student demonstrations, had been improved in this respect by amendments in the French Senate, but many researchers are still unhappy.

Hermes to win approval soon?

BRITAIN'S space industry was earlier this week still awaiting government approval of a proposed multimillion dollar contribution to the European space shuttle project, Hermes. The approval, due last Monday (1 December), is for about 10 per cent, or \$4 million, for the first phase of the space project. The preparatory phase, which began in the autumn and will take a year to complete, will cost \$40 million. The complete programme, geared to a launch in 1995-96, will cost \$1,200 million, of which France and West Germany will provide 39 and 30 per cent, respectively.

But a great deal of research is needed in the preparatory phase to tell how companies with expertise in space technology will benefit. Nearly 30 submissions on how the technology can be advanced and ex-

ploited are with the European Space Agency, which is coordinating the project.

The British National Space Centre, formed about a year ago to coordinate the British space effort, awaits a response from the government to its 15-year plan, which includes the Hermes project. The centre wants an early decision so that preparations can be made for a ministerial meeting of agency members next June.

The Hermes is a space aeroplane to be launched aboard the new European rocket Ariane V, which will be ready for its first flight in about 1994-95. The research on Hermes is expected to inspire applications in other military and civil aviation fields. New types of wings and flight systems would be two possible areas of research.

Bill Johnstone

IMAGE
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REASONS

Associated Press.

Effective yet peaceful protest in Paris.

ADEMAST, the Association Nationale Pour le Développement et la Maîtrise des Sciences et des Techniques, an association of scientists that grew up after the epoch-making 1982 national colloquium on science and technology in Paris has also strongly criticized the bill, now withdrawn after the protest. In a report published just before last week's demonstrations, ADEMAST admitted deficiencies in the universities but rejected the constant politicization of university reform.

Governments "must work with what exists" rather than attempt in each parliament to recreate the universities anew, said the scientists' associates. And it gave a strong endorsement to the system of multiannual contracts, by which the ministry of education in the previous administration was guaranteeing extra funds to universities.

These funds were available only to establishments which put in place a serious research and training policy to meet local and national needs. These multiannual contracts changed the distribution of university funds from what had been uniform "sprinkling" to real selectivity, the association claimed. But the new bill appeared to be weakening this kind of selection rather than strengthening it.

The scientists' association recommends the creation of an agency to distribute university funds selectively. The agency would be independent of the ministry, and similar to the British University Grants Committee; but again the new law moved in the opposite direction in the name of autonomy. According to ADEMAST members, French universities are still immature after the revolution of 1968; and the new law would have led to a mere counter-revolution rather than an improvement. Whether any changes at all will now occur is yet to be seen.

Robert Walgate

Chemical weapons

Study reflects Atlantic divisions

Washington

EUROPEAN and US perceptions of the value of North Atlantic Treaty Organization (NATO) chemical weapons are still very much at odds, to judge from a new joint study* by independent strategists. The Aspen Strategy Group in the United States and the European Strategy Group failed to find common ground on the key question of whether NATO's policy of modernizing chemical weapons while removing them from Europe is justifiable. And both are pessimistic about a total ban.

Chemical weapons remain a source of

deep divisions within the NATO alliance; although European countries want a treaty banning them, the United States is keen to preserve the chemical option as a means of countering Soviet first use and to decrease reliance on nuclear weapons.

Under NATO rules of engagement, chemical weapons would be used only if Warsaw Pact countries used them first. The Soviet Union has more advanced chemical capabilities than NATO, and opinions differ over their importance to Soviet war plans.

The Aspen/European study believes

that chemical weapons are probably not a Soviet "weapon of choice". But it could not agree on the wisdom of the planned modernization of the NATO chemical weapons stockpile.

Many, not least the US government's General Accounting Office, have serious reservations about the likely usefulness of the 'Bigeye' binary chemical bomb now being developed (see *Nature* 321, 717; 1986). Binary weapons are in principle safer than the single-agent weapons now in storage, because two relatively non-toxic agents do not become lethal until mixed during flight. But the Bigeye has had severe technical problems.

Unable to reach joint agreement on NATO strategy, the Aspen/European study settled instead on the face-saving formula that chemical weapons should not be a cause of alliance disunity in deterring conventional or nuclear war in Europe. The group almost tries to have it both ways by saying there is no justification for NATO acquiring a large-scale chemical war-fighting capability, but also that few believe NATO policy should allow nuclear deterrence to be the only means of deterring possible Soviet use.

The compromise seems to be that a small offensive capability would be prudent, which is what NATO has. Given that, the group could find no rational reason for removing them from Europe to the United States. The NATO plan to airlift chemical weapons across the Atlantic in a time of crisis is seen by the group as substantially limiting their deterrent value; the logistical burden in a military crisis would be very substantial.

Negotiations for a comprehensive chemical weapons ban have been in progress for nine years, with little sign yet of agreement. Verification is, of course, the stumbling block, with the United States and the Soviet Union deadlocked mainly over the question of on-site inspections.

The Soviet Union has accepted the principle that destruction of chemical weapons stocks should be monitored continually by on-site inspectors, and appears to be moving towards accepting the need for a quota of on-site inspections to verify compliance. But the United States recently hardened its position, arguing the need for a short notice (48-hour) mandatory challenge procedure.

The Soviets so far reject anything but voluntary inspections, although there is a British proposal that might break the deadlock. Under the proposal, an on-site inspection could be legally refused if the accused party provided evidence that the inspection was unjustified. The proposal fails, however, to specify the type and standard of "evidence of innocence" that would suffice.

Tim Beardsley

National Science Foundation

New centres for biotechnology

Washington

THE US National Science Foundation (NSF) plans to spend more money on shared instrumentation and multidisciplinary basic research related to biotechnology. The new programme has two strands — facilities centres to be set up in 1987 and research centres to follow a year later.

Eight million dollars have been appropriated for the development of multi-user instrumentation facilities, or 'mini-centres', in 1987. The foundation expects to make 10–15 two-year awards averaging \$500,000 each for these centres, which are being developed to meet the demands of the "consistent lobby of people [doing basic biological research] who need access to sophisticated instruments for less than full-time use", according to Thomas Quarles at NSF.

The instruments NSF has in mind include costly items such as DNA or protein synthesizers, peptide sequencers and the new nucleotide sequencers developed earlier this year. Prices vary, with the DNA machines costing between \$30,000 and \$90,000, and the peptide machines ranging between \$85,000 and \$160,000. These prices are prohibitive for many research laboratories, especially if they need the machines only occasionally.

The instrumentation sharing programme has two objectives. The first is to maximize the return on investment by attempting to ensure nearly full-time use of expensive equipment by combined groups of researchers. The second is to stimulate multidisciplinary exchanges through the common use of equipment.

The centres are not, however, designed to be regional facilities located equidistant from the researchers who will use the instruments. David Kingsbury, the assistant director for the directorate administering the programme at NSF, says he sees the instrumentation awards in 1987 going to

single departments or groups of investigators in one area.

According to Kingsbury, the success of previous regional shared instrumentation centres has been mixed, depending on the users' location. Often, the institution physically housing the equipment has received the greatest benefit, with little regular sharing with researchers elsewhere.

To qualify for support under the research centre scheme in 1988, laboratories will need to demonstrate the quality of their research programme as well as their need for equipment. The aim is to encourage people from various disciplines to work together on important problems in biotechnology. The money is expected to establish, modify or support existing multidisciplinary biological research centres, and the grants will be structured to fill gaps not financed by industry, universities or other government entities.

Kingsbury expects "everyone under the Sun" to apply for the research centre grants, and foresees joint proposals from, for example, ecologists and microbiologists and from chemists and biologists. These awards will depend on the treatment by Congress of the NSF budget for 1988, not yet published, but may be as large as \$2–4 million per centre.

NSF says that it has no preconceptions of the topics these centres should deal with except that they should be within the field of biotechnology and should not be related to diseases or the development of drugs, which are the responsibility of other agencies.

Proposals for the 1987 facilities centres are due by 1 April 1987, and those for the 1988 research centres by 1 August 1987. Awards will be made after the deciding panel meets in May or June. James Brown, the division director at NSF, says it will be "nip and tuck" to make the facilities awards within the 1987 fiscal year, which ends next September. **Carol Ezzell**

* *Chemical Weapons and Western Security Policy*, Aspen Strategy Group and European Strategy Group, 1986.

Telecommunications

Fight for Japanese network

Tokyo

As Tokyo is one of the three principal financial centres of the world and growing the world's telecommunications giants are fighting to plug their cables into Japan. But an attempt by Britain's Cable and Wireless to tap Japan's lucrative international telecommunications market has met with a solid wall of resistance.

Following last year's liberalization of Japan's telecommunications law, a consortium headed by Cable and Wireless and C. Itoh, a major Japanese trading house, declared its intention to set up an international telecommunications company that would break the monopoly held by Kokusai Denshin Densha (KDD).

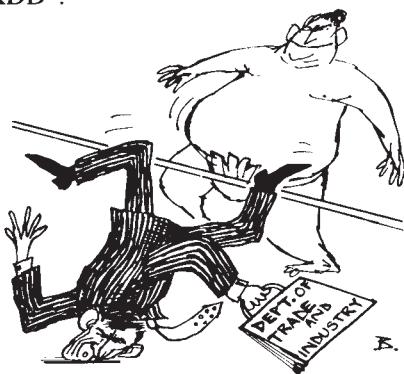
But Mitsubishi Corporation, Mitsui and Co. and Sumitomo Corporation beat the C. Itoh/Cable and Wireless consortium to establish a feasibility study company, International Telecom Japan (ITJ), in July (see *Nature* 321, 803; 1986).

Whereas ITJ plans to follow established telecommunications routes — Intelstat satellites and a submarine trans-Pacific cable (TPC-3) due to be laid by KDD and American Telephone and Telegraph in 1988 — the C. Itoh/Cable and Wireless group was expected to link up with Cable and Wireless' global digital highway, a network of submarine and transcontinental optical fibre cables connecting Western Europe, the United States and the Far East. But there has been strong opposition to the C. Itoh/Cable and Wireless plan.

Both the Ministry of Post and Telecommunications and KDD maintain that there is not enough room for a third company, and KDD says there is no need for an extra trans-Pacific submarine cable. But the most conservative estimates of Japan's international telecommunications market forecast a tripling in size to ¥600,000 million (£2,600 million) by the mid-1990s.

Taking heed of the ministry's pronouncements, the C. Itoh/Cable and Wireless group approached the major shareholders of ITJ in October and suggested a merger but their offer was turned down. And in mid-November the group formed International Digital Communications Planning Co. (IDC) with Cable and Wireless and C. Itoh the major shareholders. No sooner had staff set up its new office than Japan's minister of Post and Telecommunications, Shunjiro Karasawa, let drop a bombshell. In discussions with British Trade Secretary Paul Channon last week, Karasawa announced that although Cable and Wireless could keep its 20 per cent share in IDC (under Japanese law a foreign company can hold up to 33 per cent of a telecommunications company), the British company would be barred from management

decisions "because international communications are relevant to national security in emergency situations" and thus a foreign company could not have an influential voice or power in a "second KDD".



With ITJ expected to apply for a licence from the Ministry of Post and Telecommunications by the end of this month, Karasawa's statement could not have come at a worse time for IDC. And although one of IDC's managing directors insists that the matter is by no means closed, he also emphasized that Cable and Wireless's trans-Pacific cable is only one of the options currently under consideration by his company. **David Swinbanks**

India creates biosphere reserve

Bangalore

INDIA's first biosphere reserve, patterned after the guidelines in UNESCO's Man and Biosphere Programme, has been inaugurated at Nilgiris in South India, one of twelve biosphere reserves suggested by the Department of Environment (DOE).

It covers an area of 5,520 km² comprising the famous Silent Valley, a region with 1,500 plant species and virgin forests of western ghats spanning the states of Kerala, Tamil Nadu and Karnataka. The Nilgiris biosphere reserve, classified as a Malabar rain forest, includes 2,020 km² of minimally disturbed forests in which about 20 tribal groups have been identified.

The reserve is cooperatively managed by the three states under existing state and central legislation on conservation and preservation of forests, wildlife and environment. India has 53 national parks and 247 wildlife sanctuaries covering 100,000 km².

According to Mr Z.A. Ansari, Union Minister of State for Environment, a comprehensive national conservation strategy is being formulated and steps are being taken to improve the living conditions of tribals in the biosphere reserves.

Radhakrishna Rao

Hepatitis B

Cheap vaccine for wide use?

Washington

AN international "task force on Hepatitis B immunization" has announced plans to use a new vaccine costing as little as \$1 per dose in mass immunization demonstration projects in four developing countries. The nine-member task force, which includes several recognized authorities on the disease, aims to convince governments of countries where the disease is endemic that mass immunization, previously considered too expensive, is economically feasible.

Hepatitis B is most common in Asian countries. It causes 80 per cent of the world's liver cancer, and may be second only to tobacco as a cause of world cancer mortality, according to one task force member, Dr Richard Mahoney. As little as a year ago, a dose of vaccine cost \$30, with three doses needed for a protective course, but Cheil Sugar and Company of Korea has now undertaken to produce it for \$1.

The Cheil vaccine is of the conventional plasma-derived type and is made under licence from the New York Blood Center. The new process increases by a factor of ten the number of doses obtained for each cubic centimetre of starting plasma, by

reducing the number of harsh purification steps and starting with more potent plasma. According to Dr Alfred Prince of the centre, another task force member, the vaccine has been tested in more than 1,000 volunteers for safety and immunogenicity, and long-term effectiveness trials are, at present, under way in several countries.

Demonstration programmes will be mounted in Indonesia, Thailand, Brazil and one African country. Over three years the group expects to vaccinate 1.2 million newborn babies. Vaccinating babies is most effective because those infected before the age of three are far more likely than others to become infectious carriers.

The task force includes members coordinated by a non-profit organization, Program for Appropriate Technology in Health, which will also help governments and international organizations to implement their own programmes.

Costs for the three-year demonstration programme are estimated at \$12.5 million; a substantial part has already been promised by charitable foundations, and the health programme is "confident" that the remainder will be found.

Tim Beardsley

US biotechnology

Research centre gets fixed home

Washington

ON an unseasonably cold, blustery day last month in the Washington suburb of Rockville, the proud parents of the Center for Advanced Research in Biotechnology (CARB) held a ceremonial groundbreaking of what will some day be CARB's new home. A joint venture of the National Bureau of Standards, the University of Maryland and the local county govern-

Information technology

New institute

FINAL preparations are being made for the opening of Britain's first higher education institute devoted to the research and teaching of information technology (IT), the marriage of telecommunication and computer disciplines, supported entirely by industry and by its users.

The new institute, to be based in Milton Keynes in Buckinghamshire just north of London, has been possible through an initial tranche of £3.5 million from more than 25 companies in the IT field, in an attempt to reduce the growing shortage of IT skills in the United Kingdom.

The institute has been created in partnership with the Cranfield Institute of Technology in Bedfordshire, whose vice-chancellor, Sir Henry Chilver, has been the chief driving force behind the project.

The institute is a new dimension in British higher education and one that has provoked detectable hostility from some in established academic institutions who suspect that industrially-funded education, the preference of the Prime Minister Mrs Margaret Thatcher, will undermine the already shaky finances of the British educational establishment.

Supporters of the institute, however, consider that its curriculum will be more appropriate to the needs of an industry already desperately short of key skills, and that its existence will lighten the burden now falling on traditional education institutions, which have been unable to satisfy the appetite of industry.

The future of the institute will depend on whether it proves to be commercially viable, which is reflected in the management structure. A managing director, Dr Allan J. Fox, reporting to a board, replaces the conventional hierarchy of vice-chancellor and senate. Fox was previously deputy director (applied physics) at the Royal Signals and Radar Establishment at Malvern.

Initial areas of research are to include software engineering, parallel processing, artificial intelligence in computers and the communications of computers linked through networks.

Bill Johnstone

ment. CARB will focus initially on protein engineering and rational drug design.

For now, CARB is housed in temporary quarters in Gaithersburg, Maryland, on the campus of the Bureau of Standards. CARB director Kevin Ulmer says the centre will take advantage of the bureau's expertise in fine measurement technologies, essential to making detailed protein structural determinations. The bureau also possesses a CYBER 205 supercomputer needed to make calculations for modelling of atomic interactions in protein molecules. Ulmer says that CARB will provide the bureau with a window into biotechnology, an area in which it has not traditionally had a very large presence.

For the University of Maryland, CARB is only one aspect of a major thrust in the realm of biotechnology. The Maryland Biotechnology Institute includes centres devoted to marine biotechnology, agricultural, medical and ethical aspects of biotechnology in addition to CARB. The university's 1987 budget allots \$5.6 million to these institutes, including provision for 75 full-time positions. Rita Colwell, university vice president for academic affairs, says Maryland is a logical place to house these centres, because the 50-mile corridor between Washington and Baltimore is rich in biotechnology companies and government research laboratories.

Montgomery County, where CARB is located, is also trying to take advantage of the population characteristics of the Baltimore-Washington corridor. The county has created the Shady Grove Life Sciences Center, and is providing funds and space to both the University of Maryland (50 acres) and John Hopkins University (35 acres) for new campuses, has begun work on what will be a teaching facility for high-technology graduate programmes, and the University of Maryland has begun CARB. County officials say the idea is to compete with the San Francisco Bay area and the Boston area for biotechnology companies. Montgomery County is home for the National Institutes of Health, the National Bureau of Standards and the Food and Drug Administration. Some four dozen biotechnology companies have already located in the county.

CARB's major initial thrust — protein structure — has in just the past two years been an area of intense interest. Thomas Steitz of Yale University calls the resurgence of structural biology "spectacular". Steitz attributes the renewed interest to strides in molecular biology that have provided large quantities of molecules of interest for study. Ability to perform site-directed mutagenesis has spurred efforts to derive a theoretical understanding of how DNA sequence changes would affect

the structure of the resulting protein.

But the increased interest in structural biology means that CARB will face stiff competition for personnel and funds. CARB has offered positions to seven scientists to form its senior staff, but so far only one has accepted. Ulmer admits it is hard to persuade industry to enter collaborative projects without a full staff on board. The National Bureau of Standards and the University of Maryland will together provide CARB with about \$3 million annually, but Ulmer hopes to increase that figure several-fold through contracts and grants from both government and industry.

Raymond Salemme, who directs Du Pont's efforts in protein structure, says

Alaskan environment

Long row ahead

Washington

THE first salvo in what is likely to be the major energy debate of 1987 was fired last week when the Department of the Interior recommended leasing 1.5 million acres of the Arctic National Wildlife Refuge in Alaska for oil and gas exploration. The government believes the land lying along the northern Alaska coast could be "the most outstanding oil and gas frontier in North America". But environmental groups call the same land a unique treasure house of wildlife, and doubt whether the area can be developed without severe ecological disruption.

Since 1980, when Congress set aside 17.9 million acres of Alaskan land for a wildlife refuge, the government has been struggling over what to do about its coastal plain. Environmental groups wanted the land declared a national wilderness, putting it off limits for oil and gas exploration. But the Interior Department's report proposes a full leasing plan, arguing that industry can conduct its operation in a way that will minimize environmental damage.

The report argues that the national need for oil and gas makes development of the coastal plain essential. A 150-mile pipeline would carry oil from the eastern boundary of the coastal plain to the head of the trans-Alaska pipeline at Prudhoe Bay.

Alaskan politicians are enthusiastic about the Interior Department's plans. The Alaskan economy is in the doldrums. The government's report maintains that oil exploration in the coastal plain will reduce US dependence on foreign oil, enhance national security and bring a more favourable balance of trade.

The coastal plain is inhabited by a wide variety of fauna, including bears, polar bears, musk oxen, foxes, sheep and migratory birds. But development will have the greatest impact on the Porcupine herd of some 180,000 migratory caribou. Appro-

most industries have been disinclined to contract with start-up operations, choosing instead to launch in-house divisions. Salem believes the interest now being shown by the Howard Hughes Medical Institute in structural biology will create academic laboratories that will compete with CARB.

Interest in protein structure is not limited to the United States. Japan has created the Protein Engineering Research Institute that Ulmer says will have an annual budget of \$10 million. In Europe, John Tooze of the European Molecular Biology Organization says that more attention will be directed towards structural work at his facility.

Joseph Palca

on oil leases

ximately 272,000 acres, or 78 per cent, of the herd's core calving area lies within the section of the refuge proposed for leasing. The report acknowledges that the impact on caribou number is "uncertain" even if care is taken to avoid disrupting the herd's movements. The report cites successful industry wildlife management at nearby Prudhoe Bay as evidence that the caribou population will not be decimated.

But Susan Alexander of the Wilderness Society says the experience at Prudhoe Bay is not relevant to the coastal plain. She argues that the principal herd at Prudhoe Bay, the Central Arctic herd, is resident, whereas the Porcupine herd is migratory. The Porcupine herd is also much larger than the Central herd but has a relatively small core calving area. Both factors, Alexander says, make the Porcupine much more susceptible to environmental change. Although assistant Interior secretary William Horn has proposed leasing the calving area last, Alexander says the damage will already be done if leasing is permitted on the range.

There is a 60-day public comment period for the report that expires on 23 January, after which it will go to Congress, sometime in the spring. Congress must approve the plan, and will probably hold its own hearings.

Representative Morris Udall (Democrat, Arizona) is likely to reintroduce legislation that would convert the land from wildlife refuge to wilderness, forestalling energy exploration. Similar legislation died earlier this year. As chairman of the Interior committee, Udall will have a powerful voice in the ultimate disposition of the land.

Alexander says a "suck-America-dry-first" policy is folly, because even Alaskan oil reserves will run out eventually. The coastal plain is a unique place, she says, one "we can afford to leave until the end".

Joseph Palca

Laboratory fraud

Another damned by publications

San Diego

THE University of California is still trying to pick up the pieces after what appears to have been an extensive scientific fraud which has left the medical school at the campus here (UCSD) severely shaken. Although the case has nothing in common with that which came to light two weeks ago at the Dana-Farber Cancer Institute of the Harvard Medical School (see *Nature* 324, 197; 1986), scientists are asking if the two cases may have been caused by pressure on investigators to publish.

The now-published report of the university's investigating committee, chaired by thoracic surgeon Richard Peters, into the San Diego fraud has revealed that as many as 68 papers published by former UCSD cardiologist Robert A. Slutsky must be considered questionable. Some of them contain fabricated data.

One of the distinctive features of the Slutsky case is Slutsky's use of what the committee calls "gift authorships", the practice of listing as co-authors colleagues who had made little or no contribution to the work, who could not vouch for its accuracy and who, in some cases, were not aware that their names had been used until the papers carrying them had been submitted for publication.

According to the committee, in the period 1983-84 Slutsky (then 37) was averaging one published paper every 10 days. The committee notes that this pace of publication went unremarked.

Although some of the co-authors were at the outset unaware of the 'gift' of co-authorship, the committee says that others questioned during its year-long investigation freely admitted to the practice of listing as co-authors senior scientists who had contributed nothing more than equipment or laboratory space to the work reported. This practice, it says, is "commonplace and condoned".

As a remedy, the committee recommends that each university department should take responsibility for specifying the participation of each faculty author in the work described in every paper submitted for publication. It says that co-authors should be responsible for reviewing all manuscripts carrying their names and should be able to vouch for the accuracy of the work. During the investigation, the committee says, it summoned Slutsky's co-authors to defend the papers on which their names appeared, but some of those concerned objected. Peters says that the notion of "innocent until proven guilty" does not apply in science.

Peters also says that the problems at the San Diego medical school could and should have been discovered earlier. The failure, he says, is less in the research

enterprise than in the senior scientists who lead it. Paul Friedman, the medical school's dean for academic affairs, said that some of Slutsky's colleagues had been suspicious of the quality of his work but that they "were simply bowled over by his energy, his enthusiasm and the size of his bibliography".

Slutsky's fabrications were apparently uncovered when he was being considered for possible promotion. Radiologist Elliot Lasser set two Slutsky papers side by side and noticed that, in two overlapping data tables dealing with control animals, the numbers of animals were different but the standard deviations were identical.

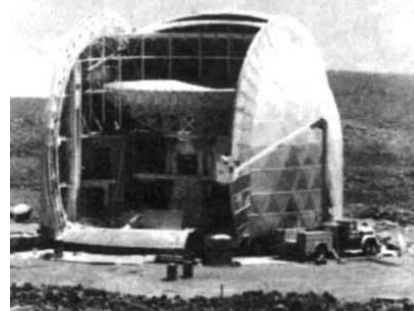
In retrospect, it has emerged that Slutsky's earlier work shows small discrepancies, as when the same data have been reused or the statistical significance of a result exaggerated. In the end, says Friedman, it emerged that the experiments simply had not been done.

Friedman says that Slutsky was caught because he did not understand statistics, and that he had survived for so long only because journals' reviewers also lack statistical expertise. Slutsky seems to have been successful because he stuck to experiments that he knew would work.

But Slutsky regularly ignored requests for supporting data, for which reason Friedman suggests that the retention of raw data should be a university requirement. He also says that young researchers should be given an estimate of what would be a realistic output of publications.

Friedman is concerned that Slutsky's unsound papers may be only the tip of an iceberg. He says that only the blatant fabrications are usually caught, so that nobody knows how many more cautious investigators cook their data in ways that escape detection. He says that the familiar use of expressions such as "dry-labbing" suggests that fraud is "not an extremely rare phenomenon".

Robert Locke



THE California Institute of Technology (Caltech) Submillimeter Observatory (shown during construction), a 10.4 metre dish that will operate at wavelengths down to 200 micrometres, was dedicated on 22 November on Mauna Kea, Hawaii. □

Science teachers

British attempt solutions

THE shortage of teachers of science, technology and mathematics in British schools prompted last week a string of proposed remedies, including the appointment of retired engineers and scientists to teaching posts, dropping the entrance requirements of universities and increasing the pool of supply teachers.

These proposals emerged at a conference in London, where representatives from 51 universities, polytechnics and colleges responded to the British government's consultation document on the shortage of specialized teachers. The government paper, *Action on Teacher Supply in Mathematics, Physics and Technology*, published in August, took the gloomy view that the present crisis will deepen as a technologically based society demands more from its workforce.

Britain needs more than 2,500 physics teachers alone to make up the shortfall, claims the Association of Science Education. At least 20 per cent of physics teaching is done by the unqualified. It is the same in mathematics and technology, where nearly a third of mathematics and a half of technology teachers are inadequately trained.

A statement signed by the universities, polytechnics and colleges says that "shortages of well qualified mathematicians, physicists, computer specialists, engineers and technologists are acting as a brake on economic growth, pushing up pay above the level of inflation and sucking the best staff out of teaching jobs in schools and higher education." The situation is aggravated by very few students applying for courses in these subjects.

The University Grants Committee, which controls the purse strings of the British universities, suggested that universities should be able to lower their admission requirements for courses for teachers in mathematics and physics; £1 million will be available in 1987-88 "for increasing the supply of mathematics, physics and CDT teachers in schools".

The Treasury announced last week that companies who second professionally qualified employees to assist education will qualify for tax relief. Industrially based mathematicians, physicists and engineers near retirement age should be encouraged to retire early and switch to teaching, says the council. **Bill Johnstone**

• British teaching problems are but a reflection of those in the United States. Last week, 90 of 126 invited research universities joined the Holmes Group; while 25 rejected the invitation. The group calls for more graduate teachers and stronger links between schools and universities.

Biotechnology companies

One falters, another forms

DESPITE its recent successful sale of stock worth \$40 million, Biogen, one of the first and best-known biotechnology companies, is not sitting pretty. Indeed, it has decided that it can no longer keep the Geneva end of the business going. Bryan Sautelle-Smith, managing director of Biogen SA in Geneva says that he is in discussion with a number of companies that might become joint partners in his company. The alternative is that Biogen SA is sold.

The financial results for the third quarter of 1986 illustrate the problem faced by Biogen NV, the parent company of both Biogen SA and Biogen Inc. in Cambridge, Massachusetts. Biogen NV has already accumulated a net loss of \$20.5 million this year, having lost almost as much in the whole of 1985. Worse still, 1986 revenue so far amounts to only \$8.5 million, half the figure of this time last year. Jim Vincent, who replaced Walter Gilbert as chief executive of Biogen just over a year ago and who is now also chairman of the company, says that the decline is mainly due to the scheduled end of payments from collaborators and to fluctuations in revenues from licensing agreements. Vincent is determined to break even as soon as possible and with few prospects of greatly increasing income in the immediate future his only option is drastically to cut costs.

Like most biotechnology companies, Biogen is faced with the fact that its revenues from sales are still small. It receives some income from the sale of hepatitis B core antigens for diagnostic test kits. And it is expecting to receive increasing royalty income from the sale of alpha interferon by the Schering Corporation, for which it developed the product. (Schering, like Monsanto, has recently sold its substantial shareholding in Biogen.) Alpha interferon received UK approval for use in hairy-cell leukaemia this year and is approved in some countries for use in the treatment of multiple myeloma and Kaposi's sarcoma, a condition often found in patients suffering from AIDS (acquired immune deficiency syndrome).

Hopes for increased sales revenue hinge in particular on the use of gamma interferon in the treatment of rheumatoid arthritis. Trials in West Germany are claimed to show its therapeutic value. Bioferon, a company jointly owned by Biogen SA and Rentschler Arzneimittel GmbH, is hoping soon to receive approval for the sale of gamma interferon for use in rheumatoid arthritis in West Germany where it has patent protection.

While Biogen savours the problems of adolescence, a new biotechnology company is launched this week in Britain.

Rising like a phoenix from the ashes left behind when Monsanto decided to axe the UK laboratories of its subsidiary G.D. Searle almost a year ago, British Biotechnology Limited starts with £2.5 million venture capital, about twenty ex-Searle staff and more than a touch of patriotism.

Fundamental to the hopes of the new company, situated in Cowley on the outskirts of Oxford, is the expertise in gene synthesis built up at Searle. Most of modern biotechnology is built on the isolation of genes and their manipulation, but British Bio-technology will synthesize and assemble parts of genes from scratch, enabling the production of hybrid proteins designed to have improved therapeutic properties. Dr Brian Richards, chairman of the new company, says that he has been careful to ensure that the patents for specific aspects of gene synthesis held by Searle do not preclude the pursuit of the general approach. A more enterprising plan is to synthesize a variety of carbohydrate groups that can be attached to the proteins, again in the hope of improving their therapeutic value.

Collaboration with established pharmaceutical companies is the initial goal of British Bio-technology. Two major projects are under discussion. One is for a novel thrombolytic agent, produced via a synthetic gene. The other is for an enzyme inhibitor made by organic synthesis, the other general technique on which the company is founded. With the help of an advisory board that includes leading British experts on AIDS, the company will examine the design of drugs against the disease and antiviral agents in general. Arthritis and bone disease are two other major targets.

The initial £2.5 million, which buys 45 per cent of the company's shares, will support the growth to about 80 staff in the next 18 months or so. Apart from the additional income from contract research, British Bio-technology also expects considerable income from the sale of their synthetic genes and carbohydrates to customers in industrial and academic laboratories.

Both Richards and Dr Keith McCullagh, the company's chief executive, are keen to tap British expertise, not least around the corner at the University of Oxford, and are proud of the 'British' in the company's title. To use it, they had to satisfy the government-run Companies Regulation Office that they were both British and pre-eminent. They will now be flying the flag alongside their neighbours in Cowley, British Leyland, the ailing remnant of the UK car industry.

Peter Newmark

Eastern Europe

Widespread shortage of energy

THE Soviet Union and its European Comecon allies face considerable problems with energy supplies this winter. This is, in part, due to the loss of generating capacity following the Chernobyl disaster. The damaged reactor No.4 is now permanently 'entombed' in concrete, reactor No.3 is still out of service and, throughout the Soviet Union, RBMK reactors have had to be temporarily withdrawn from service to make them 'operator-proof', a process which, according to Dr Valerii Legasov, will not be completed until 1987.

But Soviet hydroelectric generating capacity is also down because of a water shortage in Central Asia. Furthermore, there are shortfalls in oil, gas and coal production, due to lack of maintenance and repair work.

Last month, it was reported at a meeting of the Supreme Soviet's Commission on the Fuel and Energy Complex that the "energy shortage" due to Chernobyl and the lack of hydroelectric power would be "met" by increased efforts from the thermal generating sector. Just what "met" means in this context is not entirely clear, as Yuri Semenov, deputy chairman of the Fuel and Energy Complex Bureau of USSR Council of Ministers, predicted that electricity supplies would be "particularly difficult" in Ukraine, Central Asia and the Transcaucasus.

There are already difficulties with heat supplies in Moscow and Tashkent. Comecon countries that import electricity from the Soviet Union have had their quotas cut. In the case of Hungary, which normally imports almost 30 per cent of its electricity from the Soviet Union, instead of the hoped for 10,500 million kWh this year, there will be a cut of 500 million kWh, of which 280 million will come in the winter months. To replace this, the Soviet Union has offered 168 million m³ of natural gas, but the Hungarians are not entirely happy about the substitution. At the Comecon summit in Bucharest last month, the Hungarian Prime Minister, Gyorgy Lazar, spoke critically about "occasional disorders in the unified system" of energy supply "in the recent period" — a remark widely interpreted as referring to shortfall in natural gas supplies either due to production difficulties in Siberia, or to supplies earmarked for the Soviet bloc being diverted to Western Europe to earn hard currency.

Restriction of supplies from the Soviet Union has led to some squabbling among the Comecon partners. In Bucharest, Lazar accused unspecified "partners" of the "unsystematic acquisition" of electricity, to the danger of Hungarian supplies. This statement was taken by the Hungarians as a reference to the Romanians,

who, the Hungarians say, in early 1985 almost blacked out the entire "integrated Comecon grid" (Eastern Europe plus the Soviet Union west of the Urals) by attempting to draw more electricity than their entitlement.

Romania has recently come under fire from several East European countries on energy issues. Last year, according to the Yugoslavs, the Romanians drew too much generating capacity from the joint Yugoslav-Romanian Iron Gates hydroelectric station. More recently, the Czechoslovak party daily *Rude Pravo* accused the Romanians of failing to supply



power station parts which, the paper said, will cause delays in refitting Czechoslovak power stations and cause electricity shortages well into 1987. Despite draconian restrictions on domestic use (only one bulb, maximum 40 W per room, and no heating of rooms or water in the morning or the evening) there are still regular lengthy power-cuts in Romania.

Other Comecon countries have also announced rationing. In Bulgaria, there is a limit on the number of kilowatt-hours allowed per household per month, and a new crime, "theft of electricity", has been defined. In Poland, a major coal-producer, there is said to be "chaos" at the coal shops, and the radio carries daily announcements as to which enterprises may expect energy supplies that day.

The preferred solution to the energy problems of the Comecon bloc is nuclear energy. Chernobyl has not changed this; the only difference is that there has been a major effort by the nuclear authorities in the member countries to assure the public that the VVER reactors they use are of different type from the RBMKs used at Chernobyl, and that, in any case, additional safety measures are being taken.

A new programme of nuclear energy cooperation in the Comecon countries has now been completed, and is awaiting ratification. This will include the construction of nuclear power stations and nuclear district heating stations for cities. Twelve such heating stations are now under construction in the Soviet Union. **Vera Rich**

US defence

Biological war still a threat

Washington

A PENTAGON report* released earlier this month on the threat of biological war from the Soviet Union reflects the growing interest in the subject in US military circles. The report reiterates claims that the Soviet Union is violating the terms of the Biological and Toxin Weapons Convention of 1972. It also provides the most comprehensive evidence so far released supporting the US contention that the release of anthrax in Sverdlovsk in 1979 was related to weapons research.

Without explaining how the evidence was gathered, the report says that as much as 10 kg of dry anthrax spores was released into the atmosphere in Sverdlovsk. The report says that the scope of the clean-up, the number of victims and the involvement of the military conflict with the Soviet explanation that contaminated meat was the source of the outbreak.

The report follows the regular five-year review of the 1972 Convention that took place in September in Geneva. Pentagon spokesman Major Randy Morger says the current report has been in preparation for some time, and had been intended to coincide with the Geneva meeting but was delayed at the printing stage.

According to Brad Roberts of the Center for Strategic and International Studies, the renewed interest in biological weapons issues can be traced to a presidential commission on chemical warfare chaired by former under secretary of state Walter Stoessel. The commission said last year that the United States could not afford to ignore Soviet activities on biological weapons, and should embark on comprehensive defensive research on biological agents. This strategy, the Stoessel commission argued, would deter the use of biological arms.

Although the Pentagon report concludes that the Soviet Union is developing biological weapons, it admits that violations of the 1972 Convention are difficult to identify. Research done for medical, biological and public health reasons is "also relevant to developing disease agents for warfare purposes". But the Sverdlovsk incident, the report claims, indicates activities that cannot be justified as prophylactic, protective or for peaceful purposes as permitted by the convention.

The report offers no further data on the yellow rain controversy, explaining in a footnote that its purpose is to focus on agents of disease rather than toxin weapons.

Joseph Palca

* *Soviet Biological Warfare Threat*, Defense Intelligence Agency DST-1610F-057-86, 1986.

Nuclear risk assessment

SIR—I write about the letter of S. Islam and K. Lindgren¹. Exposure to nuclear accidents and the question whether nuclear engineering is safe or not, and to what degree, is not a question to be answered by sterile theory. The life, health and general well-being of many depend on the availability of cheap and comparatively safe energy. If silly calculations exaggerate the problems caused by mass hysteria, this is not in the best interest of an advanced technological society.

The conclusions reached by Islam and Lindgren are not only wrong but, worse, misleading for those such as the Greens in West Germany. Here are some obvious flaws.

(1) The authors write that "risk assessments which use data from operating experience are a must". I wish they had followed their own advice. What did they do? They inserted the operating experience (reactor years) of a very mixed sample and two accidents (Three Mile Island and Chernobyl) into some formulae. On such a basis, a hypothetical assessor of earthquake catastrophes would have entered an observation period of 10 years at the time when Shakespeare wrote *King Henry VI*, ten to eleven years after the "London Earthquake", Isaac Newton would have used 107 years when his *Philosophiae naturalis principia mathematica* was published and R.A. Fisher would have taken 470 years in 1950. One may repeat this *Gedankenexperiment* in relation to the catastrophic earthquakes of 1811–12 south of St Louis, Missouri. The authors should also ask themselves what their exposure calculations would have produced had the London or the Missouri earthquakes not occurred.

Backed by decades of risk assessment and using the very extensive data of the two largest reinsurance companies, I can assure the authors that a realistic probabilistic evaluation of the catastrophe potential is possible only if one has plenty of information on all accidents, from the smallest to the very largest; only then can reliable probability distributions be inferred.

(2) The authors have (generously) not counted the Windscale accident of 1957 "because it was a military reactor". What matters is the type and the parameters of a reactor, not whether it is a private or a military reactor.

(3) It is obviously unknown to the authors how large the difference is between Soviet RBMK-1000 reactors, light-water moderated reactors and other types. Accident probability depends not only on such basic differences but on engineering details, quality control and on hazards such as fire, explosion, earthquake, flood and so on, and on human parameters. No geengroc-

er would conclude from an observational sample of thousands of banana and strawberry-years that he must also sell his coconuts and almonds within a day or so to reduce loss from deterioration. Calculations that lump all types of reactors into one sample are not worth taking seriously.

Risk assessment segregates the total exposure according to parameters controlling accidents, when it is possible to correlate actual damage experience with individual parameters to find out which of them appears to be important, whether the list is complete and how much information on accidents is available. More advanced methods (*cf.* ref. 2) but even comparatively simple stochastic calculations, will show that a very large number of observations is necessary per subsample to produce reliable results.

Regrettably, I must add that I know of only a few cases where planners, designers, engineers or contractors of large and risky projects have consulted those with the largest collection of data on accidents and damage (such as specialized insurers and reinsurers).

Unfortunately, it is not generally realized that mathematical models, hypothetical reasoning or thinking carefully about accidents, using logically plausible scenarios³ and subtleties such as wondering whether to use Poisson, Pearson, Weibull or Gumbel III, are no cheap substitute for difficult, time-consuming and costly compilations of information on actual failures. The observed failure frequency of civil engineering structures, for example, is several orders of magnitude greater than the safety to be expected from calculations⁴. How large is the error margin if far more exotic projects are designed according to logical, albeit theoretical scenarios?

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2. Wald, A. *Sequential Analysis* (Wiley, New York, 1947).
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Avalanche dowsing

SIR—I am grateful to *Nature* for publishing accounts of the scientific status of the shadowy field of paranormal phenomena, an area most serious scientists deliberately ignore.

The Norwegian Red Cross Mountain Rescue Organization officially advocates dowsing as an effective method for finding victims of avalanches. This voluntary organization has about 5,000 members in the mountains during the Easter skiing season. Dowsing has been taught for more

than 10 years, under the pretence that the technique is accepted by mountain rescue organizations in other countries. Dowsing exercises have been part of the avalanche safety course taught to career officers of the Norwegian Army, and dowsing rods are part of the Army equipment for avalanche rescue.

On 5 March 1986, 16 young Norwegian soldiers died in an avalanche at Vassdalen in northern Norway. Dowsing was attempted by an Army officer in an area where seven or eight soldiers were later found dead. He got between 20 and 30 signals, of which one or possibly two pointed to a victim. He attributes the poor result to the presence of water and vegetation under the snow but is convinced the technique would work under other conditions. A central representative of the Red Cross has expressed the opinion that the dowser was not sufficiently familiar with the technique. The two positive finds made by dowsers at accidents in snow can both be associated with external visual clues. The results are thus in line with those presented, for example by Foulkes¹ and Marks², that in the absence of such clues the outcome of dowsing is no better than a series of guesses.

Fortunately, dowsing at the Vassdalen catastrophe did not interfere with the regular search activities which took place simultaneously.

Dowsing is not accepted by the Norwegian police and the Norwegian Geotechnical Institute, responsible for scientific studies of avalanches in this country, has taken a firm stand against the technique.

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1. Foulkes, R.A. *Nature* **299**, 163–168 (1971).
2. Marks, D.F. *Nature* **320**, 119–124 (1986).

Wrong dose

SIR—In your recent article "Occupational radiation: British nuclear workers cleared" (*Nature* **323**, 481; 1986), there was a major error. It was reported that for Sellafield workers, "the average radiation dose for those for whom film badge records are available is given as 124 mSv (12.4 mrem) accumulated until the end of 1983". Which dose is correct? A dose of 124 mSv is equal to 12.4 rem. 12.4 mrem is equivalent to a dose of 124 μ Sv. There is a large difference between 12.4 mrem and 12.4 rem.

T.L. KELLOGG

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- The smaller dose was that intended. Editor, *Nature*.

Images in and of the brain

It is beginning to be possible to produce images of the brain in action, but interpretation of the pictures obtained remains uncertain.

DRAMATIC pictures of the brain in action are now being obtained by optical recording using voltage-sensitive dyes and by positron emission tomography. Some of them have been published in *Nature* (Blasdel, G.G. & Salama, G. **321**, 579; 1986 and Fox, P.T. *et al.* **323**, 806; 1986); last week, A. Grinvald *et al.* (*Nature* **324**, 361; 1986) reported an optical method for recording cortical activity without the use of dyes, at least when the surface of the cortex can be made accessible.

Producing these images requires sophisticated computer processing; understanding them needs tutored interpretation, involving some of the same neural mechanisms that are the subject of the images. Problems such as these were discussed at a recent symposium* on images and understanding, which brought together people who generate images, from the arts and computer graphics, together with neuroscientists and artificial intelligence people who seek to understand the mechanisms by which the brain analyses images.

The conundrum of how the brain interprets images of the brain was illustrated by Horace Barlow (Cambridge) and Marcus Raichle (St Louis), but the implications were left unexplored. The advantage of the new cortical imaging techniques is that the activity of many neurons can be monitored at once, in the intact brain, so as to give a much more complete picture of the spatial organization of neuronal responses to particular stimuli than could previously be had by painstaking reconstruction, and, with some guesswork, from single-unit recordings. Microelectrode recording is, of course, not superseded by the glamorous new technology, for it remains the best way of characterizing the responses of individual neurons. Rather, the synoptic methods add a new dimension by making it possible to see all the neurons activated at once by a particular stimulus; they provide functional maps of the brain.

What do such maps reveal? It has long been known that the brain itself contains a map of the external world — in several areas of the cortex, there are detailed point-to-point, though not necessarily proportional, representations of sensory fields or of the body surface. A consistent finding is that the world is represented many times over, both within a single area and in several regions; different aspects of

the same information are then processed in parallel channels.

Colin Blakemore (Oxford) suggested that one reason for such maps is to bring common functions to adjacent points for efficient processing of information. This may be true for orientation and ocular dominance (the basis of depth perception) in the striate area of the visual cortex, as Blasdel and Salama's voltage-sensitive dye maps suggest. But in the same region, cells responding to colour and direction of movement are physically intermingled yet functionally quite separate and they project to separate secondary visual areas (Hubel, D.H. & Livingstone, M.S. *Nature* **315**, 325; Shipp, S. & Zeki, S. *Nature* **315**, 322; DeYoe, E.A. & Van Essen, D.C. **317**, 58; 1985). The organization at these relatively low levels in the processing hierarchy is probably a sorting process through which the correct combinations become addressed to multimodal processing centres higher up the system.

All the maps of the world are symbolic, the maps of the brain included. Reading them must be learned, just as people must learn to interpret geographical maps. Because it is still early in the development of the new imaging technology, the symbolic conventions are not well established and the viewer has to work hard to translate grey-scale or false-colour images into patterns of cortical activity. What is more, the published images are static snapshots of activity patterns at different times.

One of the most difficult features of the brain to capture and analyse is the pattern of dynamic change in the activity of and interaction between its constituent neurons. Because animals and the world around them are rarely still, the sensory input to the brain is constantly changing. Even when one looks at a static image, the attention shifts to focus on different aspects — and appreciation of the same image on different occasions is tempered by physiological and psychological states.

Others know of the difficulty. The art historian, Ernst Gombrich, pointed out how difficult it is to represent a sequence of movements pictorially without recourse to symbols or verbal explanation. Pictorial instructions are therefore rarely universally comprehensible. A clear example of how the interpretation of symbols depends on cultural context and hence on learning was provided by Monica Parker, choreologist to the Royal Ballet, London. In the Benesh notation for recording

movements, a basic diagram representing a simple position with arms outstretched at shoulder height would look very different if adopted by an Indian classical dancer rather than by a Western dancer. The details that give the position its character will depend on the form of expression inherent in the tradition of the interpreter.

Although many of the visual images we create and enjoy are static, the perception of movement is a basic and important aspect of visual analysis. In primates, the motion-processing channel starts in the retina but directional sensitivity is first displayed by neurons in area V1 of visual cortex and further elaborated in the motion-analysing area, called MT.

Neurons in MT project to association areas including temporal cortex where, in monkeys, David Perrett and colleagues at St Andrews have recorded from many visually driven cells selective for aspects of body movements. Their results begin to give a glimpse of how the brain reconstructs complex images. Each cell responds to limited views of the body and limited sets of movements, for instance, to a body facing and moving towards the viewer or to a particular articulation of a limb. Many do not depend on absolute form, but respond similarly to a monkey, a human being or with a pattern of dots, as long as it is moving correctly. Although these higher-order cells combine information about motion, form, colour and depth, each still responds only to selected features of the object. Specific recognition or perception of the body as a whole must be the product of collaboration between many cells. Where and how this occurs for any image, simple or complex, is one of the unsolved mysteries of neuroscience.

As a highly visual species, how human beings perceive the world is bound to be a challenging subject; most research on sensory processing and perception has focused on the visual system. But it was for an artist, Liliane Lijn, to point out that ultimately our perception of the world is multisensory; visual images are reinforced by sound, smell and touch. How and where in the brain all the parallel channels carrying the various aspects of all the stimuli converge are unanswered questions. Beyond that still are our conceptual capabilities, defined by Richard Gregory (Bristol) as the marshalling of ideas on a long timescale to develop long-term strategies, generalizations and abstractions.

Jennifer Altman

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Mathematics

One hundred per cent proof

Ian Stewart

MATHEMATICIANS are a funny lot. They operate in a world of mental abstractions, and many of them appear to have little interest in anything outside it. They pursue abstruse problems on a basis of aesthetic taste, rather than directing their attention at explicit practical goals. Despite this otherworldliness, the results of their deliberations can have enormous practical impact, but that is not my target here, which is the following. To the eternal frustration of their colleagues in neighbouring disciplines, mathematicians are without exception obsessed by the belief that nothing in mathematics is true unless it has been proved, not just beyond reasonable doubt, but beyond unreasonable doubt. Why?

The seamen of ancient Corinth knew that if you tie an overhand knot in a rope, then it cannot be removed except by untying it. Mathematicians remained unconvinced until 1926, when Kurt Reidemeister came up with with an argument that went beyond mere plausibility and established as a mathematical fact the existence of knots that cannot be untied.

Nobody in his right mind would deny that a closed curve in the plane, which does not cross itself, divides the plane into two pieces — the inside and the outside of the curve. Mathematicians were sufficiently sceptical that the question was for many years a well-known and important unsolved problem, stated explicitly by Camille Jordan in 1887, who gave a lengthy, intricate proof. Several other distinguished mathematicians, unhappy with the details, gave their own proofs, but they all contained gaps or mistakes, and the first rigorous proof was given by Oswald Veblen as late as 1905.

In 1852 Francis Guthrie, a student at University College London, wrote to his brother Frederick about a tantalizing little conundrum that he had stumbled across while colouring a map of England. "Can every map drawn on the plane be coloured with four (or fewer) colours so that no two regions having a common border have the same colour?" Experiment rapidly suggests that the answer is yes. But can it be proved? At first this four-colour problem looked fairly easy, the kind of thing that any competent professional ought to be able to polish off with one hand tied behind his back. In 1879 Arthur Kempe, a barrister who belonged to the London Mathematical Society, published a clever argument that seemed to settle the matter. But the problem turned out to be rather more subtle. In 1890 Percy Heawood found a mistake in Kempe's argument, and showed that the best that could be

salvaged was a proof that five colours suffice. For almost a century this tiny gap, between five proved and four conjectured, remained unbridgeable. Finally Kenneth Appel and Wolfgang Haken (*Ill. J. Math.* 21, 429 and 567; 1977) produced their celebrated proof that four colours are always enough. Their method involved testing some two thousand maps for a property called reducibility, which took about 1,000 hours on a fast computer. There are still a few die-hards who do not consider this to be a proof at all, because it uses a computer, and the odd rumour of errors still goes the rounds, but most people are convinced.

My concern here is not the nature of proof. That is a very interesting question, but it would take us too far afield. I also hasten to add that during the initial phase of mathematical creation, rigorous proof is usually left to one side until the basic ideas have crystallized out. My question is: why are mathematicians so hard to



satisfy? In other sciences the evidence required to accept a theory is not a logical proof that nothing else could possibly be the case. It is experimental evidence that the predictions of the theory seem to be correct. The stronger the evidence, the happier people are with the theory; and they have no difficulty in operating under the slight possibility that one day the theory will turn out to be false after all. Not seeking absolute truth, they are content with not finding it.

Not so long ago, mathematics was felt to be exceptional. Its truths, it was said, really are absolute. After a good dose of Gödel, the general opinion changed. Mathematics is only as true as its foundations, and it is impossible to prove that the foundations are 100 per cent solid. Mathematicians have learned to live with that. The natural sciences went through something rather similar but a good deal earlier: eighteenth century fears that scientists knew so much about the Universe that they were doing themselves out of a

job also turned out to be unfounded.

But mathematicians still insist on very high standards of logical rigour, and are unimpressed by experimental evidence, even when it appears to be virtually watertight. For example, perhaps the most important open problem in mathematics is the Riemann hypothesis, stated in 1859 by Bernhard Riemann. It is known that the zeta-function $\zeta(z) = 1^{-z} + 2^{-z} + 3^{-z} + \dots$ is zero when the complex number z is a negative even number. The hypothesis is that any other zeros have real part equal to $1/2$, that is, they are of the form $z = 1/2 + iy$. J. van de Lune and Herman te Riele in Amsterdam have just shown, by computation, that the first 1.5×10^9 zeros satisfy this hypothesis. Nobody considers this strong enough to be a proof.

Why do mathematicians take scepticism to such a lunatic degree? There are several reasons. One is that there have been many occasions when intuition or strong experimental evidence has turned out to be misleading. An extension of the Jordan curve theorem states that when a curve divides the plane into two pieces, the inside piece is topologically equivalent to the inside of an ordinary round circle drawn in the plane, and the outside piece is topologically equivalent to the space outside that circle. The natural three-dimensional analogue of this is the hypothesis that when a surface topologically equivalent to a sphere is embedded in space, then the space is divided into two regions, topologically equivalent to the inside and outside of an ordinary round sphere. This is very plausible and natural. Unfortunately, it is not true. The Alexander horned sphere shows the outside space can get very messy indeed.

When a young research student called Stephen Smale first told his supervisor he had proved that you can turn a sphere inside out without introducing creases, he was told to go away and find the mistake. Smale is now one of the world's leading mathematicians, and his theorem is both true and famous.

In the theory of prime numbers the number of primes less than a given number x , denoted $\pi(x)$, is approximated by various analytic formula. One is the so-called logarithmic integral $\text{Li}(x) = \int \frac{dx}{\log x}$. The ratio of $\pi(x)$ to $\text{Li}(x)$ tends to 1 as x tends to infinity, so $\text{Li}(x)$ is a good approximation to $\pi(x)$. What about the error? 'Experimental evidence' shows that $\pi(x)$ is less than $\text{Li}(x)$ for x up to about 10^9 or so. This many successful experiments might be considered conclusive. Mathematicians do not see it that way, and neither did S. Skewes who proved in 1933 that $\pi(x)$ must be greater than $\text{Li}(x)$ for some x no bigger than $10^{10} \cdot 10^{34}$, where (for typographical convenience) $^{\wedge}$ denotes exponentiation.

This points to a major problem with experimental evidence in mathematics. Evidence based on only a finite number of

cases is really pretty thin, and that is usually all the evidence you can get. One billion is a tiny number compared with a googolplex ($10^{10^{100}}$), and most numbers are bigger still. Almost all numbers are immense. The proportion of numbers smaller than Skewes' apparently monstrous number, for example, is zero. Indeed in number theory many estimates on sizes of things grow like, say, $\log \log \log x$. This is 34 (using logs to base 10 for convenience) for Skewes' number. Tiny.

So experience shows that plausible intuitions are not to be trusted, and that numerical evidence is not worth the computer paper it is printed on. The problem is compounded by the way mathematics is constructed. It is like a huge house of cards: theorem piled upon theorem, shored up by lemmas and corollaries, with axioms deep in the foundations acting like concrete pilings and hoped-for conjectures floating up in the clouds, unattached to the main building. If one tiny part is faulty, the whole edifice may collapse. I don't mean this too literally: if a mathematician makes a mistake, it seldom has implications that serious — maybe a balcony falls off. There are internal checks and balances. But to maintain the structural integrity of mathematics it is important not to admit shoddy components.

A standard method of proof, which is extremely powerful and therefore very popular, is 'proof by contradiction'. To prove a statement X the mathematician starts out by denying X, follows up the consequences, and eventually arrives at something that contradicts either itself or some known theorem. This shows that the denial of X is false, hence X itself is true. It is rather like offering a gambit in chess in the hope of recouping greater gains later; but, in the words of Godfrey Hardy, the mathematician offers "the whole game". Imagine the dangers of using a result that has not been given a decent proof, however plausible it may be, in the course of such an argument. It does not bear contemplating, and mathematicians therefore try very hard not to contemplate it.

Another reason for wanting rigorous proofs is that their absence is a sign that something important is not understood. The Jordan curve theorem, for instance, led to algebraic topology. This is central to today's mathematics, and is currently producing useful payoff in various scientific areas, notably gauge field theory. On the whole, algebraic topology is not the sort of technique that gets invented in a direct attack on a practical problem. It is not like calculus, say, whose need becomes apparent the moment the real-world problem is formulated in mathematical guise. Obsession with proof is not just pedantry: it is a major source of new ideas and methods.

In science, the experimental method is paramount. It evolved primarily as a way of counteracting the natural human ten-

dency to believe something because one wants it to be true. If scientists allow themselves the luxury of believing a theory because it is pretty, or conforms to their political prejudices, then they cease to do effective science. If an engineer wants to make sure that his bridge will not fall down he does not worry about the aesthetics of the design, or the colour: he checks that the design conforms to acceptable tolerances and he makes sure that the bridge

is built to specification. In the same way, a mathematician dare not believe a plausible theorem just because he would like it to be true, and he cannot place great faith in even extensive numerical evidence because there are good reasons to assume that may be misleading. Proof, and proof alone, is the safeguard. □

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Cancer chemotherapy

Progress in understanding multidrug resistance

George R. Stark

RESISTANCE to a wide range of drugs is often found in human tumours that have relapsed after initial regression in response to drug therapy¹. An understanding of this phenomenon would be important in the search for more effective chemotherapy. On page 485 of this issue², Gerlach *et al.* report a striking similarity between the amino-acid sequence of P-glycoprotein, a membrane protein of relative molecular mass 170,000 (170K) whose overaccumulation can lead to multiple drug resistance, and the *hlyB* gene product, a 66K membrane protein that mediates active export of the protein haemolysin from *Escherichia coli*. This homology is sufficiently extensive to suggest functions for the P-glycoprotein. Two other groups have recently reported complete complementary DNAs predicting sequences of P-glycoproteins from mouse³ and human⁴ cells; the results are very similar to those obtained by Gerlach *et al.*².

Overaccumulation of P-glycoprotein results from amplification of a small family of closely related genes in multidrug-resistant cells^{5,6} (for example, cells resistant to anthracyclines, vinca alkaloids, podophyllotoxins and dactinomycin but not to bleomycin nor most alkylating agents and anti-metabolites). Amplification of many genes occurs at a high frequency in cells of an advanced tumour stage and amplification of oncogenes undoubtedly contributes to malignant progression (see ref. 7 for review). It is likely that a small tumour containing around 10^9 cells already contains a few thousand cells in which the P-glycoprotein genes have been amplified, even before therapy⁷. Worse still, treatment with some anti-tumour drugs or radiation can increase the rate of gene amplification⁸ and thus increase the fraction of multidrug-resistant cells.

Overexpression of the P-glycoprotein genes is almost always a result of amplification and is the predominant cause of multidrug resistance in current examples. It is clear that there is a small family of

closely related P-glycoprotein genes^{6,8} and by screening a complementary DNA library from normal mouse cells, Gros *et al.*⁹ obtained a clone for one of the P-glycoproteins that includes the entire coding region. When this sequence was placed in an expression vector and transfected into drug-sensitive hamster cells, selection with adriamycin revealed multidrug-resistant colonies in which only the introduced mouse DNA was amplified. This result shows that overexpression of a single member of the P-glycoprotein gene family confers resistance.

P-glycoproteins are relatively scarce in the membranes of most cells but can be increased by 100-fold or more in multidrug-resistant cells. How can an overproduced membrane protein mediate resistance to a diverse collection of drugs, often unrelated in structure and function? Recent work shows that the drugs accumulate less in resistant cells, probably as a result of an increased rate of efflux rather than a decreased rate of influx, suggesting that the P-glycoproteins may pump many different small hydrophobic molecules out of cells^{10,11}. Support for this idea comes from other studies using a derivative of vinblastine that can be photoactivated and appears to bind directly to the P-glycoprotein^{12,13}. Note, however, that there are other cases of resistance to single drugs in which, for example, specific pathways for drug influx have been lost¹⁴, and also well-known mechanisms of resistance that have nothing to do with transport⁷.

Analysis of the similarity between P-glycoprotein and *E. coli* transport proteins supports and greatly extends the efflux model of multidrug resistance. Sequence analysis of the complementary DNAs reveals that the P-glycoproteins consist of a tandem duplication of a more basic structure^{3,4}. Gerlach *et al.*² show that the homology with the haemolysin transport protein extends over about 230 amino acids at the carboxy terminus of P-glycoprotein. From work with monoclonal anti-

bodies, it is known that the carboxy terminus is found on the cytoplasmic side of the plasma membrane¹⁵. By comparison with homologous regions of other transport proteins, it seems likely that part of this cytoplasmic domain functions as a nucleotide-binding site, probably using ATP to provide the energy for active efflux²⁻⁴. As the sequence of P-glycoprotein consists of a tandem repeat, each molecule would provide two such sites.

In anticipation of a direct study of the function of P-glycoproteins for their role in multidrug resistance, two major mechanisms have been considered²⁻⁴: (1) direct efflux of hydrophobic drugs through a P-glycoprotein pore, perhaps involving other components of the membrane; or (2) protein-mediated efflux in which the drugs are first bound to a carrier protein which is then expelled from the cell. The latter possibility is suggested by the close homology to the haemolysin transport system, which is known to function in this way. The homology also raises questions about the normal function of P-glycoproteins.

Knowledge of the structure and functions of the P-glycoproteins should lead to new attempts at tackling multidrug resistance in patients. It may be possible to take advantage of the exposure of part of the overproduced P-glycoprotein on the cell surface to target cytotoxic antibody conjugates to these cells after the bulk of the tumour has been destroyed by conventional chemotherapy. As the details of the transport mechanism become better defined, it will be possible to consider the design of specific drugs which can interfere with it; compounds have already been discovered that interfere with efflux of drugs from multidrug-resistant cells¹⁶.

An understanding of the mechanism of multidrug resistance presents the possibility of much greater clinical benefit from the enormous effort that has in the past been put into developing and testing chemotherapeutic agents. □

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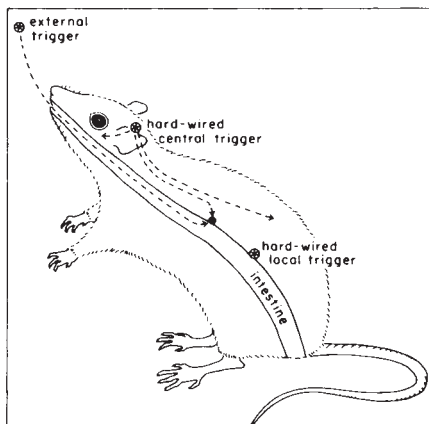
Developmental physiology

Hard-wired local triggering of intestinal enzyme expression

Jared M. Diamond

NORMAL development requires that each gene express itself at the right time and in the right cell. Is proper timing triggered by signals external to the animal, or is it 'hard-wired' within the animal (see figure)? If triggering is hard-wired, is the trigger local, within the tissue where expression will occur, or is it central, for example, in an endocrine gland whose hormones control many peripheral tissues? A recent report by K. Yeh and P. Holt (*Gastroenterology* 90, 520; 1986) demonstrates the existence of a hard-wired local timer for sucrase expression in rat intestine, which will be a useful model for the identification of timers in other systems.

Infant rats are normally weaned from a diet of maternal milk to solid food around 25 days after birth. Starting around day 18, intestinal activity of lactase (which



Gene expression in developing rat intestine may be pre-programmed by a local timer in intestinal cells themselves; pre-programmed by a centrally released timer (for example, a thyroid or pituitary hormone); or not programmed at all but instead triggered by an external event.

digests the milk sugar lactose) declines, whereas sucrase (which digests sucrose in solid food) first appears (Henning, S. A. *Rev. Physiol.* 47, 231; 1985). These changes might be externally triggered by weaning and the resultant change of solutes entering the intestinal lumen: experimentally delaying the age of weaning, for example, is found to delay the decline of lactase.

The changes might also be under hard-wired central control by hormones that modulate many other developmental changes around the time of weaning. (Pituitary, thyroid and adrenal hormones affect development of intestinal lactase and sucrase, as well as that of many other

functions.) Could a hard-wired local timer contribute as well?

Yeh and Holt transplanted isografts of mid-intestine from donor rat pups 0 or 5 days after birth to under the skins of newborn (day 0) host rat pups. Some of the grafts survived and exhibited normal migration rates of differentiating enterocytes along the crypt/villus axis. They found that the control of expression in the isografts differs between lactase and sucrase.

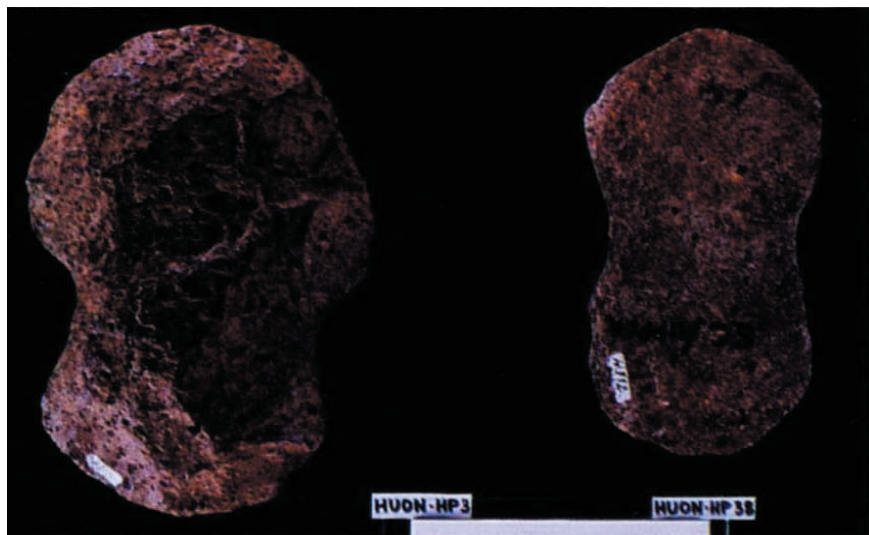
Isograft lactase does not begin to decline until after donor age 28 days (host age 28 or 23 days), although lactase in the host intestine begins to decline as usual around 18 days. Thus, the change in luminal solute input, which was experienced by the host intestine but not by the donor intestinal graft under the host's skin, is an important signal for turning off lactase expression. In agreement with this conclusion, the normal decline in lactase is delayed in bypassed intestinal loops (Tsuboi, K. *et al.* *Gastroenterology* 80, 1550; 1981) and is accelerated in rat pups fed an artificial milk formula (Yeh, K. & Holt, P. *Pediatr. Res.* 19, 963; 1985).

Sucrase activity begins to rise simultaneously around day 18 in donor and host tissue when day 0 donor tissue is transplanted into day 0 hosts. But when day 5 donor tissue is transplanted into a day 0 host, the isograft begins expressing sucrase when the host is only 13 days old and the donor tissue 18 days old. At this time the host tissue expresses no sucrase, which does not appear until 5 days later when the host reaches an age of 18 days.

Thus, donor sucrase expression is not triggered externally by luminal solute inputs (from which the donor intestinal graft was cut off), nor by a hard-wired central timer in the form of host hormones (which would have triggered host and donor expression simultaneously). Nor could the normal absence of sucrase expression at day 13 result from a systemic inhibitor present then, as the graft does express sucrase at a host age of 13 days. Instead, there must be a hard-wired local timer within the donor intestinal tissue that turns on sucrase expression at a tissue age of 18 days. In agreement with this conclusion, sucrase expression begins at the normal time in bypassed intestinal loops and in rat pups from which the pituitary, adrenal or thyroid gland is removed. □

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Discovery of waisted axes in New Guinea



THE recent discovery of waisted axes such as those pictured above indicates that human inhabitants were present in Papua New Guinea more than 40,000 years ago. On page 453 of this issue, Les Groube and his co-workers describe the excavation undertaken (at the south-east end of the Huon Peninsula in New Guinea) and the artefacts discovered there. The archaeological site consists of a series of coral terraces of varying ages, the waisted axes being localized to regions believed to be more than 40,000 years old. This dating was confirmed by thermoluminescence analysis of resident quartz particles. These new data suggest that New Guinea was inhabited at a relatively early stage in the Pleistocene settlement of Australia from the Indonesian-Indochina region, when it and Australia were connected by a land bridge. □

Albert Szent-Györgyi (1893 – 1986)

ALBERT Szent-Györgyi, one of the most remarkable biochemists of our century, died in Woods Hole, Massachusetts, on 22 October. The son of a Hungarian well-to-do farmer, he grew up in a comfortable and cultivated household. He had started to study physiology when he was called up for military service in World War I. In the post-war upheavals he wandered from one laboratory to another, learning new techniques from Michaelis and others, until he settled in Holland, first in Leiden and then with H.J. Hamburger in Groningen. He became deeply interested in biological oxidations, especially the catalytic systems involving succinate, fumarate, malate and oxaloacetate. He recognized their wide general significance in metabolism, and his work represented an important preliminary step that helped toward the later demonstration of the total tricarboxylic acid cycle by Hans Krebs.

Szent-Györgyi also became interested in vegetable respiration, and detected a reducing agent, present in many plant juices, which he noted because it caused a slight delay in the progress of oxidations catalysed by peroxidases. That he took notice of this slight delay, which many investigators might have ignored, was characteristic. When Hamburger died, Szent-Györgyi had to find a job elsewhere; by great good fortune Sir Frederick Hopkins, who had followed his work with interest,

invited him to Cambridge and obtained a fellowship for him. Here he succeeded in crystallizing the reducing agent from the juice of oranges, lemons and cabbages, and from adrenal glands.

About this time (1932) an enlightened Hungarian minister of education recognized Szent-Györgyi's ability and invited him back as professor of medical chemistry at the University of Szeged. A young Hungarian, J. Swisbely, who was familiar with the assay for vitamin C, came to work with him. It was soon apparent that the crystalline reducing agent was indeed vitamin C (ascorbic acid). Szent-Györgyi found that Hungarian paprika was extraordinarily rich in this substance, and was able to prepare crystals in kilogram quantities, which he distributed to other researchers throughout the world. It was for his work on ascorbic acid that he received the Nobel Prize in 1937; the citation also noted the importance of his work on the role of dicarboxylic acid anions in biological oxidation systems.

Next he became involved in the study of muscle contraction and the proteins of muscle. On extracting myosin with salt solutions by the standard procedures, he noted that, if the extraction was prolonged, the extract became considerably more viscous, although the total amount of protein extracted was not much greater. This led to the recognition that another

protein, later called actin, was associated with myosin. Szent-Györgyi's young co-worker, F.B. Straub, purified actin, while he himself investigated the ATP-induced contractility of actomyosin threads.

Szent-Györgyi carried out this work, which opened great new vistas in muscle biochemistry, during World War II under constant danger from the Nazis, who had become aware of his contacts with the British Secret Service. He was placed under house arrest, and escaped seizure by the Gestapo only by taking refuge in the Swedish Legation in Budapest. When the Nazis broke into the Legation, he fled just in time, and he and his wife went into hiding until the war ended.

When the Soviet armies arrived, Szent-Györgyi and his family were protected and well cared-for on personal orders from Molotov, and later he was brought to Moscow for two months. But he became disillusioned and outraged by the character of the Soviet regime and the atrocities committed by the Soviet armies in Hungary. Although personally well treated by the authorities, he found it impossible to continue the work of his laboratory and so departed for the United States, where he settled at the Marine Biological Laboratory in Woods Hole.

Always ready to strike out into new fields, he became fired with interest in the concepts of quantum mechanics, although he never claimed to have mastered (or even to have actually grasped) the mathematical complexities of the subject. Along these lines, he wrote a small monograph on what he termed 'submolecular biology'.

His major preoccupation during the last decades of his life, however, was with cancer. His views on the origins of cancer differed widely from those of almost all other workers in the field, and government grants for his support, although available in limited amounts, were quite inadequate to support research on the scale that he considered necessary. He continued research with a small group, but his last years must have been embittered with a sense of frustration.

He was always, especially in his earlier years, a man of infinite jest; he loved to announce his ideas and discoveries in a manner that could lead the unwary to believe that he was talking nonsense; but they soon discovered that there was a very solid basis for it all. Like many jesters, he had a deeply tragic vision of the world; he was appalled by the follies of its rulers, teetering constantly on the brink of destruction. Somehow he still managed to cherish hope. Scientist and humanist, he was a unique and unforgettable man.

John T. Edsall

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Evolutionary ecology

Toxins and grazing resistance

Peter D. Moore

ONE of the most useful things about plants, where human beings are concerned, is their capacity to resist the attentions of grazing organisms by the production of deterrent chemicals. These secondary compounds are among the most important controls on grazing insects¹ and by happy chance provide a rich source of drugs, for example, caffeine². Considerable effort is now being directed towards the study of plant/grazer interactions as a potential source of pesticides and drugs, and simply as an area in which co-evolution is most graphically illustrated. Some recent investigations seek to demonstrate the importance of secondary compounds in a range of lower plants, including cycads³, lichens⁴ and algae⁵, as well as in higher aquatic plants⁶.

Cycads are well known as plants toxic to vertebrates⁷, but the North American cycad *Zamia floridana* is fed upon by the brightly coloured caterpillar of a Lycaenid butterfly, *Eumaeus atala*. It has been supposed that the red and yellow coloration of the larva indicates that it sequesters glycosides for its own defence, as is the case in the monarch butterfly⁸, but the study of this particular plant/animal inter-relationship has been hampered by the extreme rarity of the insect. A captive breeding programme has now provided

sufficient larvae to permit analysis and Rothschild, Nash and Bell¹ report that both the caterpillars and the adult insects do indeed contain the glycoside cycasin and are therefore toxic to predators. They also emit a pyrazine-like odour which presumably deters enemies. Their conspicuous coloration, also a deterrent, is unusual among the Lycaenids, suggesting that the association between cycad and

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Unpalatable meals? Larvae of Eumaeus atala florida (photo-graph courtesy of D.C. Lees, London Butterfly House).

butterfly has been a long one. It would be interesting to know whether the cycad receives any benefit from the relationship, perhaps in the form of pollination.

Down the scale of evolutionary complexity, secondary compounds in the lichens are also implicated in herbivore deterrence. Lawrey⁴ examined the behaviour of slugs when presented with a selection of lichens and found that they exhibit a distinct preference for certain species — perhaps it would be more accurate to say that they definitely avoid the unpalatable species. Even acetone extracts of the thalli of avoided species are effective in deterring the predators. The fact that it is avoidance of the distasteful rather than preference for the attractive which operates is shown by the higher levels of nitrogen, phosphorus, potassium and calcium in the avoided species. Those lichen species, which should have been most alluring to the slugs, are in fact the most distasteful because of their heavy load of secondary compounds.

In marine algae, there are secondary compounds which could also be involved in grazing resistance. For example, many species of the red alga genus *Laurencia* have sesquiterpenes, compounds which are often involved as insect deterrents in higher plants⁹. There are even parallels to the terrestrial grazers which have an immunity to toxins or even store them

for their own protection against predators. The sea hare *Aplysia californica*, a marine mollusc, is thought to do this with the sesquiterpenes of *Laurencia pacifica*¹⁰. But it is evident that not all the grazing effects of herbivores on algae are necessarily harmful to the plant. Zooplankton may stimulate phytoplankton growth rates by local enrichment of water with ammonia¹⁰; Connor and Quinn¹¹ propose that growth of some microalgae is stimulated by the mucus of grazing limpets.

But Crews and Selover⁵ now suggest that *Erythrocytis saccata*, a red algae semi-parasitic on *L. pacifica*, may take up sesquiterpenes from its host and retain them in the same concentration in its own thallus. They have not demonstrated actual transport, but any other source of this particular suite of compounds seems unlikely. Perhaps the parasite gains some degree of predator immunity from the sequestered defence materials.

It is not only the algae among aquatic plants that are well equipped to deal with the attentions of grazers. Ostrofsky and Zettler⁶ show that the same is true of many aquatic higher plants, such as some species of the pondweed genus *Potamogeton*. Possession of deterrent secondary compounds may account for the low level of herbivore attack on such species. *P. crispus* is particularly well supplied with alkaloids and the authors consider that the concentrations present (0.2–0.5 mg per g dry weight) are adequate to account for the general lack of grazing on such aquatic macrophytes. But some terrestrial mammals are known to graze on aquatic plants, for example the moose, which derives most of its sodium requirement from summer grazing in shallow lakes¹². Perhaps this animal, like the sheep¹³, has some degree of resistance to the alkaloids contained in the aquatic macrophytes and thus takes advantage of a relatively underexploited resource.

□

100 years ago

I wish to suggest a possibility, which if true will considerably alter our views of the facts of chemistry. Let us take the electrolysis of hydrochloric acid.

At present we state the facts thus: Every molecule of hydrochloric acid consists of one atom of chlorine and one atom of hydrogen, the chlorine atom weighing 35.5, the hydrogen atom weighing 1. On passing a current, each molecule is split into these two atoms, each atom carrying a unit charge of electricity.

It is not just possible that we may some day state: A molecule of hydrochloric acid consists of one molecule of hydrogen weighing 1 combined with 35.5 molecules of chlorine each weighing 1. On electrolysis, the chlorine atoms are split from the hydrogen atom, the chlorine atoms each carrying unit charge of electricity, and the hydrogen atom carrying 35.5 charges of electricity. If this is the truth, then all the atoms of the elements are of the same weight, and probably are made of the same "stuff", and we have only two things which condition the properties of the atom — its electrical charge and its electric potential, and Mendelejeff's table becomes a statement of the periodic relationship between these.

From *Nature* 35, 132; 9 December 1886.

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Galactic evolution

Do globular clusters belong?

Virginia Trimble

Do the globular star clusters really belong to the galaxies they now inhabit, in the sense of having formed in and with them? Yes, according to some of the results presented at a recent symposium*, and no, according to others. The issue arises because these clusters are a bit like dinosaur teeth — small hard things that could have survived a long time under harsh conditions. Thus, they might, alternatively, have formed separately before their host galaxies or as part of other systems that amalgamated to make those galaxies. The issue is important because the globular clusters, unlike dinosaur teeth, are not just very old, but are actually the oldest subjects in the Universe available for study at close range. They ought, therefore, to tell us something of when and how galaxies and stars began.

Globular clusters have so far been seen in about 40 galaxies (spirals, ellipticals and irregulars) and studied closely in half a dozen. The 125-or-so clusters known in our own Milky Way contain, for instance, about 1 per cent of the stars of the spherical halo. Evidently, indicators are needed to show whether the clusters resemble the stars around them more than they resemble each other, and whether their properties (individually or as populations) are correlated with those of their host galaxies. Resemblance and correlation would suggest formation *in situ*, and the opposite would suggest a more complex process.

The evidence, unfortunately, is contradictory and rather evenly balanced. Starting with our own Galaxy, the globular clusters (according to R. Zinn, Yale University) do generally resemble the other halo stars in their distribution of numbers as a function of radius, in their average heavy-element abundance and its gradient with galactocentric distance, and in their dynamical properties (net rotation, velocity dispersions in three dimensions and average orbital eccentricity). Differences exist, however, in the periods of variable stars as a function of their composition (G. Wallerstein, University of Washington) and in the metallicity distribution, in the sense that the field stars have a larger representation at very high and very low values than do the clusters (J.B. Laird, University of North Carolina).

Other indicators that the clusters 'belong' to their hosts are: the likelihood that galaxies more massive than the Milky Way, including M31, NGC5128 and M87, have redder, more metal-rich clusters (F. Fusi Pecci, University of Bologna; H.C.

Harris, US Naval Observatory; J. Huchra, Center for Astrophysics, Cambridge, Massachusetts), though the high value for M87 was disputed by J. Nemec (University of British Columbia); the two-standard-deviation result that central (massive, cD) galaxies in clusters have more than their fair share of globulars (W.E. Harris, McMaster University); and the undisputed fact that gas-rich galaxies, including M33 and the Magellanic Clouds, unlike the Milky Way and similar spirals, are still producing massive clusters (though not quite such massive ones as the true, old globulars; E. Olszewski, Steward Observatory; C. Christian, Canada–France–Hawaii Telescope).

In contrast, several other observations suggest that the clusters did not originate with the galaxies they now inhabit. First, the number of clusters at a given luminosity, the function $N(L)$, seems to be much the same, a gaussian with the same width and a peak at $M_v = -7.4$, in nearly all galaxies examined; at any rate, the assumption that this is so leads to a consistent distance scale out at least as far as the Coma cluster (D.A. Hanes and D.G. Whittaker, Queen's University, Ontario; L.A. Thompson and F. Valdes, Institute for Astronomy, Hawaii). Second, the clusters in M31 (Fusi Pecci) and M87 (Huchra) do not show the kind of gradient of abundance from centre to periphery seen in the Milky Way. Both items suggest that the clusters did not know where they were going to end up when they formed.

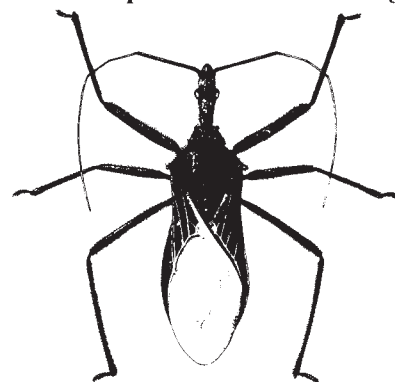
Finally, and most striking, the clusters in some galaxies differ from the non-cluster stars at the same radial position in a number of related ways. The cluster populations are less centrally condensed than the underlying star light, scaling as $r^{-2.5}$ rather than as r^{-3} in M87, M49 and other large elliptical galaxies¹, where r is the distance from the centre of the galaxy concerned. They are more spherically distributed around the galactic centre than the background stars in disk galaxies including NGC3115, 4594 (the Sombrero) and 7814 (Harris). They are bluer than the star light at the same positions both in M87 (J. Cohen, Caltech, confirming earlier work²) and in several dwarf ellipticals³. And they display a larger velocity dispersion than the other stars at the same radius in NGC5128⁴ and M87⁵. The net impression is that the clusters constitute an older, more primitive population, the bluer colours resulting from lower metal abundances; and the greater extension, sphericity and velocity dispersion representing a lesser degree of collapse and dissipation.

This could mean that the clusters are pregalactic and primordial^{6,7}, or that they formed as part of larger units that merged to make modern galaxies (R.B. Larson, Yale University). But it could also mean that the globulars formed *in situ* as galactic halos collapsed, their population statistics having been gradually modified as the most vulnerable clusters were torn apart tidally during close approaches to the galactic centres (J.P. Ostriker, Princeton University; M.J. Rees, Institute of Astronomy, Cambridge; D. Chernoff, Cornell University). Tidal disruption would systematically remove central clusters (hence the shallower density profile) and ones on eccentric orbits. The remaining circular orbits then give the impression of increased velocity dispersion at any given off-centre point. To account for the colour difference, the metal-rich clusters must also have been systematically removed, which is also plausible, at least in galaxies with radial abundance gradients. Apparently, then, all the scenarios can still accommodate all the data and no-one suggested unambiguous clues that might be sought in the near future. □

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Two new species of the genus *Pristhesancus* are described by M. B. Malipatil in a recent issue of the *Australian Journal of Zoology* (34, 601–610; 1986). The illustration shows a male specimen of *Pristhesancus nigritus*, dorsal view. The scale bar represents 3.2 mm. This bug,



found in Northern Australia, is generally black with two pale distal antennal segments. Like other members of the order Heteroptera, this specimen has two pairs of wings and mouthparts modified for piercing and sucking. Many heteropterous insects are vectors of plant and animal diseases. □

* International Astronomical Union Symposium No.126, Globular Clusters. Harvard University, 25–29 August 1986.

Clinical pharmacology

New hopes for the treatment of coronary heart disease

Gilbert Thompson

A RECENT 6-year survey¹ of more than 350,000 middle-aged men in the United States confirms that both total mortality and death from coronary heart disease are strongly correlated with the concentration of cholesterol in the blood. Death rates rise steeply once serum cholesterol exceeds 6.5 millimoles per litre, and values above that level are found in 15 per cent of the population. In the United Kingdom, new epidemiological data² indicate that there are twice as many men with this degree of hypercholesterolaemia. In most instances this problem can be managed by dietary means, but a significant minority of individuals will require drug therapy. A new agent that reduces the concentration of cholesterol in the blood is described by A.H. Underwood and colleagues on page 425 of this issue³.

The suggestion that hypercholesterolaemia causes coronary heart disease is confirmed by the atherogenic effect of increased levels of low-density lipoprotein (LDL) cholesterol and the protective effect of high levels of high-density lipoprotein (HDL) cholesterol. Drugs that lower LDL and increase HDL are used to treat familial hypercholesterolaemia. People with this condition (0.2 per cent of the population of the United Kingdom) have a propensity to accelerated atherosclerosis and premature death from coronary heart disease. M. Brown and J. Goldstein discovered that this disorder represents a genetically determined defect of specific cell-surface receptors for LDL and have elucidated the structure and

function of the receptors.

As illustrated in the figure, the LDL receptor provides a path of entry for exogenous cholesterol into the cell and reduces endogenous cholesterol synthesis through down-regulation of the rate-limiting enzyme β -hydroxy- β -methylglutaryl coenzyme A (HMG CoA) reductase⁴. The number of LDL receptors expressed and the activity of this enzyme in the cell have complementary roles in maintaining cholesterol homeostasis. This process is disturbed in familial hypercholesterolaemia where defective function of the receptors results in diminished uptake from plasma and decreased degradation of LDL, processes which normally take place mainly in the liver. The LDL receptor is a glycoprotein of relative molecular mass 160,000 (160K), the gene for which is on chromosome 19, whereas HMG CoA reductase is a 97K glycoprotein, the gene for which is on chromosome 5 (ref. 5). Several mutations of the gene encoding the receptor have been described⁶, any of which can give rise to familial hypercholesterolaemia, but no mutations of the gene encoding HMG CoA reductase have been identified.

Hitherto the ability to stimulate hepatic LDL receptors has depended on the use of insoluble anion-exchange resins such as cholestyramine. These resins bind bile acids within the intestine and thus impair their re-absorption into the blood. To make good this loss, the rate of conversion of cholesterol to bile acids in the liver increases, the consequent need for ad-

ditional cholesterol being met partly by increased expression of LDL receptors and partly by a compensatory rise in cholesterol synthesis.

The LDL-lowering effects of cholestyramine can be markedly enhanced by concomitant administration of a new class of drugs that block cholesterol synthesis by competitively inhibiting HMG CoA reductase. Like cholestyramine, these compounds promote receptor-mediated LDL catabolism but are much easier to take. They include mevinolin (lovastatin) and synvinolin, and look set to revolutionize the treatment of heterozygous familial hypercholesterolaemia^{7,8}. Unfortunately homozygotes, with fewer functional LDL receptors, respond less well.

The new work of Underwood *et al.*³ in this issue is based on the well-known fact that thyroid hormones lower serum cholesterol. In attempts to use this effect therapeutically, avoiding undesirable side-effects by administering D-thyroxine instead of L-thyroxine, even D-thyroxine proved dangerous, causing cardiac arrhythmias when given to patients with coronary heart disease, and its use has been largely discontinued. Underwood *et al.*³ have discovered a thyromimetic agent, SK&F L-94901, which has significant cholesterol-lowering properties in animals. Their data suggest that this compound is equipotent to triiodothyranine in its effect on the liver but one thousand-fold less active in its effect on the heart. Although its development is still at an early stage, the outlook seems promising. The mechanism of the cholesterol-lowering effect presumably involves enhancement of receptor-mediated LDL catabolism, which also occurs during treatment of hypothyroidism with L-thyroxine⁹. The possibility that it stimulates selectively hepatic LDL receptors is intriguing but if, like thyroid hormones, SK&F L-94901 also increases HMG CoA reductase activity¹⁰, it might be necessary to administer it with an HMG CoA reductase inhibitor. Such a combination could be just what is needed if diet-resistant hypercholesterolaemia is to be treated as routinely and effectively as hypertension, as the evidence¹ suggests it should. □

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

A cultured fibroblast showing binding to receptor, endocytosis and lysosomal degradation of LDL, resulting in hydrolysis of protein to amino acids and of cholesteryl ester (linoleate) to free cholesterol. This leads to down-regulation of HMG CoA reductase and LDL receptors. Excess free cholesterol undergoes esterification to cholesteryl oleate by the enzyme acylcholesterylacyltransferase (ACAT). (Reproduced from ref. 11 with permission from M.S. Brown and J.L. Goldstein).

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Astrophysics

Big, repulsive boson stars

Robert Walgate

THERE is normally little need to remark that all familiar stars — even quark stars and neutron stars — are made of fermions, particles of half-integral spin whose quantum statistics forbid them to enter the same quantum state. The result of this well-known fact is the 'degeneracy pressure' which keeps the fermions in different states like the electrons in an atom, and prevents even quite massive, relativistic stars from collapsing under gravity. But is it possible to conceive of a stable gravitating star made of bosons, the integral-spin particles which can form a single quantum state of, in principle, unlimited intensity? The theoretical Universe is, after all, beginning to be filled with exotic particles of many kinds from the bosonic 'superpartners' of the quarks and leptons to Higgs bosons and 'ghost' matter; it is conceivable that if boson stars exist then some of the supposed missing matter of the Universe could be in the form of such objects. In fact, as M. Colpi, S. L. Shapiro and I. Wasserman report (*Phys. Rev. Lett.* **57**, 2485; 1986), the answer seems to be yes.

Initial calculations supported by refined work with general relativity led R. Ruffini and S. Bonazzola (*Phys. Rev.* **187**, 1767; 1969) to suggest that boson stars would be possible. But by astronomical standards these early calculations gave the stars a tiny mass, of the order of the square of the Planck mass divided by the boson mass (around 300 million tons, or 10^{-19} solar masses for 1 GeV bosons), and a radius of the order of the Compton wavelength of its constituent bosons (1 fm or so for 1 GeV). It was difficult then to argue for a real astronomical role for such objects. The new work of M. Colpi *et al.* shows that the earlier conclusions — which assumed non-interacting bosons — can be drastically modified if the bosons have the slightest repulsive force among them.

The new work thus demonstrates that boson stars of in principle any mass might be assembled, depending on the interaction strength and mass of the constituent bosons. For example, it follows that a unit interaction strength (of the same order as the strong interaction) and a 1 GeV boson mass would lead to a boson star not of 1 fm, but of 100 m radius, and to the corresponding relativistic maximum mass of 0.1 solar masses. If the bosons were the putative axions of 10^{-5} eV mass, the star would be a crazy 10^{28} times bigger; whereas smaller interaction strengths could produce an object of, for example, the mass of Jupiter. This range indicates at least that boson stars must now be considered candidates to join the observers' menu of

theoretical exotic stars.

Why is the interaction important? Essentially because any repulsive force of the order of magnitude of, or greater than, the gravitational force can keep the star buoyed up against gravity. If the force is short-range, at some critical mass gravity will overcome the repulsion and collapse will ensue; but the critical mass will be sensitive to the parameters of the repulsion. As the gravitational force is so weak (10^{-39} times the electromagnetic interaction between protons, for example), the assumption of even an extremely tiny force can have a dramatic effect. This is what Colpi *et al.* observe and develop in a full general-relativistic calculation.

Whatever the details of this calculation, the quantities and orders of magnitude involved are themselves illuminating. To set a baseline, the authors first assume non-interacting bosons. The radius, R , of a relativistic star (one near gravitational collapse, so that gravitationally bound particles have velocities near the speed of light) of such bosons will be of the order of the Compton wavelength of a single relativistic boson. Thus $R \sim 1/m$ in natural units, where m is the mass of the boson. (In natural units Planck's constant and the speed of light are taken to be unity. In such units, with masses in GeV, roughly the proton mass, lengths are approximately in femtometres.) But as the orbits are relativistic, R must be near the Schwarzschild radius GM , where G is Newton's gravitational constant and M is the mass of the 'star'. Combining these equations we find $M \sim 1/Gm$. Now we can replace G by its expression in terms of the Planck mass — the mass M_p such that the gravitational self-coupling GM_p^2 is roughly unity, and we find that the mass of the 'star' is $M \sim M_p^2/m$. This reproduces the bones of Ruffini and Bonazzola's result. The mass density of such a star would be M/R^3 , or M^2/m^2 , a gargantuan 10^{38} times nuclear density for 1-GeV bosons.

The first appropriate expression of Colpi *et al.* for the energy density of the interacting bosons, if ϕ is the boson field (or wavefunction, to use a more familiar name), is $m^2\phi^2 + \lambda\phi^4$. Here the first term approximates the mass and kinetic energy of a relativistic particle and the second is the assumed contact interaction term, which would arise, for example, if the bosons repelled one another by the exchange of a heavy intermediate particle. The question now is, what do we mean by 'small' interactions being important? By small we must mean that $\lambda\phi^4 \ll m^2\phi^2$, in other words that the interaction energy is

much less than the kinetic or mass term. To extract λ from this inequality it is necessary to know the value of ϕ , the boson amplitude in the star. But the mass density of the star is $M_p^2 m^2$, as shown above, which must equal the boson-energy density. If λ is 'small' we can set the latter equal to $m^2\phi^2$. As a result, we see $\phi \sim M_p$, so that finally we get the limit on λ that $\lambda \ll (m^2/M_p^2) = Gm^2$. This means the self-interaction must be much weaker than gravity if its effect is to be negligible.

The next problem is to develop the theory with large λ , but the problem (S. L. Shapiro, personal communication) is that there are two dimensionless quantities to deal with in the calculations: λ and m/M_p . Hand-waving arguments thus fail to give any guidance, and it is necessary to solve the full relativistic equations. The resulting maximum boson star masses work out, for 'large' λ , to be around $M \sim 0.1 M_\odot \lambda^{1/2}/m^2$, with $M_\odot$ the mass of the Sun, rising as expected with the repulsion but falling as the square of the boson mass.

This is not the end of the story, however: refinements are still needed. Colpi *et al.* would like to deal with Higgs bosons, key elements of current unified field theories which in the empty (pre-inflationary) vacuum have an energy density much like that assumed above but with an attractive term (λ is negative). This leads to a condensation of Higgs field in the vacuum at the minimum of the energy density function (rather like the transition to superconduction in a superconductor). The condensate shortens the range of the weak interaction, giving the intermediate vector bosons the corresponding mass. Oscillations of the Higgs density are also allowed, and the corresponding waves are, in principle, observable, through wave-particle duality, as Higgs 'particles'. These are bosons, and could form boson stars — Higgs stars. Inside such stars, the Higgs field (which creates all particle masses) will vary, and the most intriguing physical possibilities arise. Unfortunately, Colpi *et al.* have so far been unable to solve the appropriate equations, which involve cubic as well as quartic terms, but work is in hand.

The group must also include the possibility of gauge forces such as colour and electromagnetism amongst the bosons. So far, Colpi *et al.* have assumed the particles to be neutral to a level equivalent to that of Ruffini and Bonazzola's neglect of possible λ terms. If the bosons were gauge charged, but on average neutral, the result would be an attractive force and the boson stars would undoubtedly collapse. So it will be necessary to consider non-neutral stars, those with a net gauge charge. Work on such stars, including the full Higgs terms, is now under way (S. L. Shapiro, personal communication). □

Robert Walgate is European Correspondent of Nature.

Plant molecular biology

Tailoring crop improvement

Chris Lamb and Leona Fitzmaurice

GENE manipulation techniques are likely to result in great improvements in crop production. Successful first steps towards introduction of disease resistance and herbicide resistance in plants were reported at a recent meeting*.

Cross-protection, inoculation of plants with mild strains of viruses, is often used to reduce losses due to more virulent strains, such as tobacco mosaic virus. To elucidate the molecular mechanism of resistance to tobacco mosaic virus infection, a chimaeric gene containing a cloned complementary DNA of the virus coat protein under the control of the cauliflower mosaic virus 35S transcript promoter has been introduced into tobacco cells on a tumour-inducing plasmid of *Agrobacterium tumefaciens*. The transfected plants produce high levels of the virus coat protein (R. Beachy, Washington University, St Louis; ref. 1). Protection from the virus, manifested as a marked delay in the appearance of symptoms, is dosage-dependent and can be partially overcome by inoculation with naked viral RNA. The number of primary sites of infection may be reduced when the virus is encapsulated by the coat protein, and the limited amount of protection to naked viral RNA suggests that there is a second mechanism affecting viral replication or spread. It will be of interest to see whether this strategy for engineering protection will be useful in other plants.

There has been progress in improving resistance of some plants to the broad-spectrum herbicide glyphosate, which inhibits the enzyme 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS). A mutant bacterial enzyme which is insensitive to glyphosate inhibition confers some resistance to the herbicide when expressed in the cytoplasm of transgenic plantlets^{2,3}. The plant enzyme is chloroplastic, and by expression of a chimaeric gene comprising transit peptide sequences of the small subunit (*ssu*) gene of ribulose biphosphate carboxylase fused to the glyphosate-insensitive bacterial gene, the resistant EPSPS can be made to accumulate rapidly in chloroplasts of transgenic plantlets (G. della Cioppa, Monsanto, St Louis; ref. 4). These plantlets are 1,000-fold less sensitive to the herbicide than those in which the transgene product accumulates in the cytoplasm.

Transgene regulation and protein targeting will be crucial for effective engineering, and new data indicate that 5'

flanking regions are not the only place where *cis*-acting gene regulatory sequences act. In petunia, the ribulose biphosphate carboxylase small subunit is encoded by a family of eight genes that exhibit markedly different levels of expression. Analysis of the expression of transgenes comprising 5' flanking, coding and 3' flanking regions of different *ssu* genes indicates that sequences within the gene and 3' flanking region have a major impact on expression (C. Dean and J. Bedbrook, Advanced Genetic Sciences, Inc.). Similarly, in chimaeric gene constructs containing the chloramphenicol acetyl transferase (*cat*) reporter gene and various promoters, the first intron from the maize alcohol dehydrogenase-1 (*Adh-1*) gene has a great impact on expression in electroporated maize protoplasts (V. Walbot, Stanford University). Expression of *cat* increased nearly 200-fold with the weakest promoter tested, 60-fold with the *Adh-1* promoter, and only 7-fold with the cauliflower mosaic virus 35S promoter, the most efficient promoter tested.

Future prospects depend on the development of an increased repertoire of gene transfer methods and systems, as well as the identification of plant genes that are agriculturally significant targets for manipulation. It is striking that only one plant receptor has so far been cloned — the photoreceptor phytochrome, involved in morphogenetic and tropic responses^{5,6}. Two isoforms of phytochrome have been identified in etiolated plants. This polymorphism, as well as differences identified between phytochromes in etiolated and in green plants⁷, could have considerable functional significance. The different polypeptides seem to arise from differential expression of members of a small gene family, although alternate translation of a single transcript, as well as post-translational modification of the proteins, may also be involved (P. Quail, University of Wisconsin, Madison). The *au^w* mutant of tomato exhibits many features of etiolated plants, and protein blot analysis indicates that the level of a phytochrome polypeptide is very low in the mutant. This system offers the potential for studying the relationships between structure and function of this receptor by examination of the effects of specific mutations engineered *in vitro* on phytochrome levels in transgenic plants following re-insertion of the modified genes, and may provide opportunities for precise engineering of changes in day-length requirements and response to shading of target crop plants. *Arabidopsis thaliana*, the common wall

cress, possesses several attractive features (ref. 8 and discussed in *News and Views*⁹). Its generation time of 4–5 weeks, the ease with which it may be propagated in large numbers, its lack of repetitive sequences and small genome size (about 70,000 kilobase pairs)¹⁰ are advantageous for the rapid identification and cloning of mutated plant gene sequences. C. Somerville (Michigan State University) produced three *Arabidopsis* plants resistant to sulphonylurea by screening 300,000 mutagenized seedlings¹¹. In collaboration with J. Smith and B. Mazur (DuPont), a yeast acetolactase synthetase gene was used to identify the gene encoding the *Arabidopsis* enzyme in a genomic library generated from one of these sulphonylurea-resistant plants. Somerville also identified *Arabidopsis* mutants that are defective in lipid biosynthesis¹². Mutants for many of the biosynthetic steps, including most of the desaturase steps, can be identified by gas chromatographic analyses of leaf tissue derived from mutagenized plants. When these data are combined with two-dimensional gel analyses of proteins, it will be possible to identify proteins that are differentially expressed in the mutants and to clone the genes of this pathway.

P. Ahlquist (University of Wisconsin, Madison) described the designed modification of brome mosaic virus for use as a vector¹³. Extremely high levels of expression can be achieved with this gene transfer system. Milligram quantities of virion coat protein are produced in infections with the wild-type virus. With engineered constructs, *cat* activity already has been obtained which is five- to fifteen-fold greater than that achieved with tumour-inducing plasmid-based vectors, and may be further increased by control of messenger RNA and/or protein degradation. Future potential advantages of this viral vector are the broad range of monocot and dicot hosts as well as effective gene transfer to vegetative cells of perennial crop plants. □

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* Tailoring Genes for Crop Improvement: An Agricultural Perspective 24–28 August 1986, University of California, Davis, USA.

BSF-2 is not just a differentiation factor

SIR—An interesting point has escaped Hirano *et al.*¹ in their report of the sequence of BSF-2, a B-cell differentiation factor. The point is that the sequence of BSF-2 is identical to that recently described by Haegeman *et al.*² of the State University of Ghent for a molecule called 26K. This molecule was originally described as a byproduct of interferon- β_1 (IFN- β_1) produced in human fibroblasts^{3,4}, and called IFN- β_2 after investigators at the Weizmann Institute detected antiviral activity associated with the molecule⁵.

In both laboratories the primary structure of the 26K/IFN- β_2 molecule has been determined by molecular cloning of the complementary DNA^{2,4,5}. But the question of whether the natural 26K/IFN- β_2 product possesses antiviral activity has remained a matter of controversy³. Hence, its true biological function has remained in question.

A first hint at an answer to this question came from the observation both in the laboratory of J. Content at the Pasteur Institute in Brussels and in our own laboratory that the mRNA for the 26K/IFN- β_2 molecule is induced in fibroblasts by interleukin-1 (IL-1)⁶. This suggested that the molecule might be one of the mediators of the multiple biological effects of IL-1. In an extensive search for biologically active substances released from fibroblasts by treatment with IL-1, Van Damme from our laboratory, together with J. Van Snick of the Ludwig Institute in Brussels discovered that fibroblasts exposed to treatments that induce appearance of the mRNA for 26K/IFN- β_2 , release a potent growth factor for B-cell hybridomas and plasmacytomas^{7,8}. In an effort to demonstrate whether this hybridoma/plasmacytoma growth factor (HPGF) is identical to the 26K/IFN- β_2 molecule, these investigators found that the mRNA for HPGF hybridizes to a cDNA probe of 26K/IFN- β_2 ⁸. Subsequently, HPGF was purified to homogeneity and, in collaboration with R. Simpson of the Ludwig Institute in Melbourne, its amino terminus was sequenced. This sequence matches exactly that predicted from the 26K cDNA clones and allows us to identify exactly the cleavage point between the signal peptide and the mature protein.

Recently there has been renewed interest in the possibility that the molecule may have direct antiviral activity⁹. Two cytokines, IL-1 and tumour necrosis factor (TNF- α), which are both able to induce the mRNA for the factor^{6,9}, also possess antiviral activity that can be neutralized by antisera against interferon- β . The question is whether these antiviral effects are mediated by IFN- β_1 or IFN- β_2 .

By using the B-cell promoting effect as an assay system, we have succeeded in preparing an antiserum that reacts with 26K/IFN- β_2 but not with IFN- β_1 . This antiserum fails to neutralize the antiviral effect of IL-1 and TNF (J. Van Damme *et al.*, unpublished data). In contrast, sera that are specific for IFN- β_1 and do not neutralize the HPGF activity of 26K/IFN- β_2 , do neutralize the antiviral effects of IL-1 and TNF¹⁰. This clearly demonstrates that IFN- β_2 does not play a role in the antiviral effect of IL-1 or TNF.

In conclusion, the substances known as BSF-2, IFN- β_2 , 26K and HPGF are one and the same. The substance certainly qualifies as a B-cell differentiation and growth factor but has little, if any, antiviral activity.

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Plant tumour induction

SIR—In Conrad Lichtenstein's otherwise excellent short comment on tumour induction by *Agrobacterium tumefaciens*¹, he omitted to mention the biological context of the bacterium. Wounding is a prerequisite for tumour formation, the basis for which is that wounded cells release inducers, such as acetosyringone, that activate the *vir*-region of the Ti plasmid of the bacterium, and thus facilitate its T-DNA transfer. *A. tumefaciens* occurs in the rhizosphere, and has been demonstrated to bind to plant cells. This implies that the bacterium is attracted to plants, but the biochemical basis for this has been left unexplained.

Intrigued, we set out to investigate chemotaxis in *Agrobacterium*. Using the methods of Adler^{2,3}, we have demonstrated that *A. tumefaciens* is capable of chemotaxis towards a range of phenolic plant wound exudates⁴ including vanillyl alcohol. Crucially, we have discovered that chemotaxis towards the acetosyringone is determined by the Ti-plasmid. Interestingly, the chemotactic optimum occurs at 100 nM acetosyringone, some 100-fold lower than the concentration giving maximal *vir*-induction.

This suggests a biological system in which acetosyringone, at low concentrations, acts as a chemoattractant for *A. tumefaciens*. The bacterium thus moves

up the concentration gradient towards the wounded plant cells. Then at higher concentrations, acetosyringone causes *vir*-induction, and ultimately T-DNA transfer.

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An alternative view of the origin of life

SIR—In his *News and Views* item on the discovery of enzymatic activities in RNA molecules, Gilbert goes on to "contemplate an RNA world, containing only RNA molecules that serve to catalyse the synthesis of themselves"¹. But what would be the source of energy in this RNA-run world? Another, and more basic question is, all these RNAs to what end? Are we interested in finding mere self-replicating machinery as an answer to the origin of life, or are we looking for a prototype of the self-propagating form as it exists in an organism?

At the simplest level, life is a massive molecular network that is able to gather a few substances to make usable energy and more molecules of different types that permit the replication of genetic material, which contains the information to run the network. The genetic material, the encoded proteins and the intermediary RNAs have received ready recognition from those engaged in the study of origin of life and its evolution; the tiny metabolites whose numerous linear, branched and cyclical conversions constitute the network, have received next to no attention. This is like embarking on the study of a kingdom and concentrating only on the king's family.

Enzymes can conceivably arise as the result of the formation of appropriate sequences of nucleic acids by random associations of nucleotides; but what function (and hence what selection value) can any of them have if there is no substrate to act upon? And, where does the substrate come from and what happens to the product? Meaningfulness demands a full pathway; but a pathway by itself is as meaningless as an enzyme in isolation. Pathways have to be able to interact with each other. The hypothesis of the formation of life by random associations of nucleotides and selection suffers from the need for prior and continual (for who knows when the gene encoding the proper

enzyme might arise?) existence of the substrate molecules.

The 'central dogma' asserting the one-way transfer of information from DNA-to-RNA-to-protein has been challenged, apart from reverse transcriptase, by the proposition of reverse translation^{2,3}, and impressive evidence exists that anticodons and their corresponding amino acids show good stereochemical fits (see ref. 4). These findings deserve to be kept in view in any formulation on the origin of genes. It is conceivable that amino acids arranged around a prebiotically-formed stable molecule (all contained in the 'soup' trapped in the self-assembled lipid bilayer) became linked to form a miniprotein. If the miniprotein had catalytic activity for the stable molecule, this 'substrate' would be converted to a new molecule, setting in motion the formation of a pathway. Branching of pathways can be visualized as two distinct types of arrangement of amino acids around a molecule, generating two enzymes for the same substrate. In time the minienzymes could be reverse-translated and reversed-transcribed to minigenes. Their integration would form the genome, providing an explanation for the clustering of functionally related genes in bacteria.

The origin and evolution of life is the most basic problem of biology. Its elucidation demands the widest permissible latitude in thinking. Is it not time that we did away with the dictates of dogmas and labels like 'heresy'?

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The kingdoms of organisms

SIR—Having been concerned with the proper definition of eukaryote kingdoms¹⁻⁵, I wish to comment on the controversy over prokaryote kingdoms between Lake^{6,7}, Woese⁸ and colleagues^{9,10}. Three distinct questions are at issue: phylogeny, the chopping up of phylogenetic trees to make taxa, and the ranking of taxa. As Woese rightly insists, only phylogeny is a matter of objective science; the others, however, are not merely semantic⁸ but questions of taxonomic judgement—that is, a mixture of art and science¹¹.

None of Lake's three trees⁷ is inconsistent with the validity of Archaeobacteria as a taxon. He confuses phylogenetics and classification in dismissing as irrelevant those characters he calls "three-and-one"; as if characters unique to all vertebrates

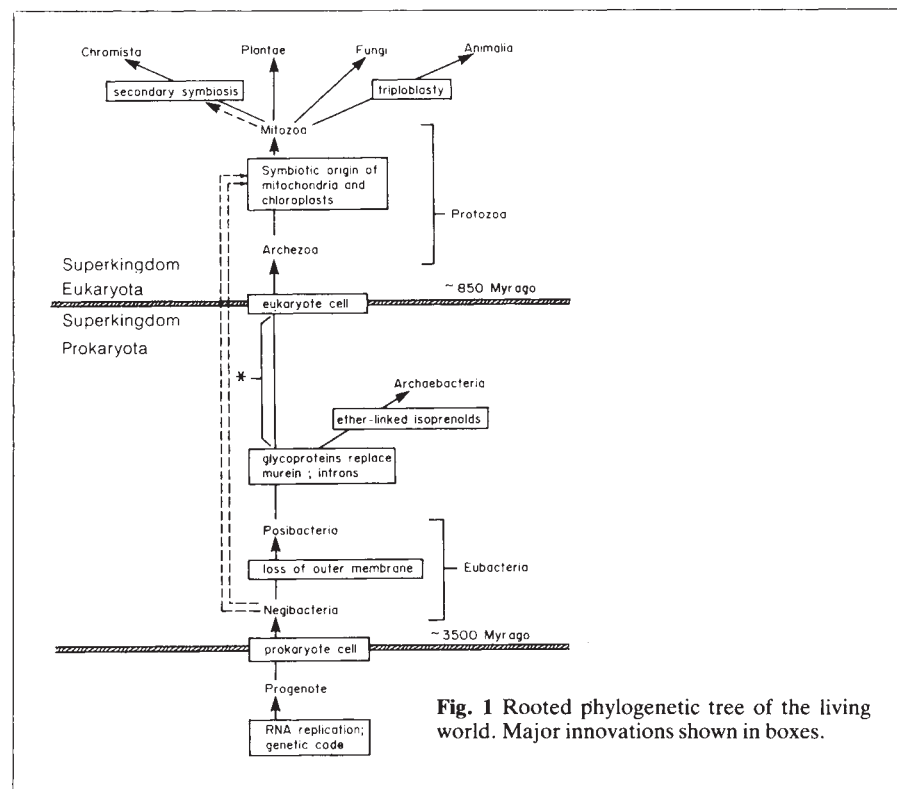


Fig. 1 Rooted phylogenetic tree of the living world. Major innovations shown in boxes.

are irrelevant to the validity of the taxon Vertebrata because they are irrelevant to overall animal phylogeny. Such characters are the most important of all in defining taxa. The distinctiveness of the wall and membrane chemistries of archaeobacteria and eubacteria and their congruence with rRNA and tRNA sequences justifies the subdivision of prokaryotes into two major taxa of equal rank, the Archaeobacteria and Eubacteria. This classification does not "fit all trees"⁷; it would be invalid if either taxon were shown to be polyphyletic, which Lake has not done.

The characters favoured by Lake are relevant to the subdivision of Archaeobacteria into lower taxa and to which archaeobacterial phenotype is closest to their nearest eubacterial relative. But his discussion of the distribution of the arginine deiminase pathway is flawed by ignoring evolutionary loss: repeated independent loss of characters in different lineages is common in evolution and is the most parsimonious explanation for the patchy distribution of arginine deiminase in bacteria. Because loss of enzymes is easy, one must not give too much taxonomic weight to the mere absence of a pathway of metabolite; many differences between Halobacteria and Methanobacteria can be attributed to the loss of ancestral proteins by the latter. The differences demand separation at least at the level of class but not at the level of kingdom or subkingdom. Similarities in RNA polymerases, antibiotic sensitivities and rRNAs make it sensible to rank Methanobacteria and Halobacteria as subphyla within a single phylum (=division), for which I propose

the name Halomebacteria. I suggest that the sulphur-dependent archaeobacteria ('eocytes') be ranked as a separate phylum (=division), the Sulfobacteria. This is compatible with all four 'prokaryote' trees.

The ranking of the taxa Eubacteria, Archaeobacteria and Eukaryota has also been insufficiently carefully considered. Woese and Fox¹³ referred to them as "urkingdoms"; this term, with no status or rank in the Linnean hierarchy, was chosen to avoid begging the question as to their taxonomic rank. Unfortunately numerous authors have carelessly substituted the Linnean term kingdom. But eukaryotes are universally subdivided into several kingdoms, commonly four¹⁴, and more recently five³⁻⁵: Animalia, Fungi, Plantae, Protozoa and Chromista. Therefore Eukaryota must be ranked as a superkingdom^{1,3}, not a kingdom. The subdivision of the living world into two major structural cell types (eukaryote and prokaryote)¹⁵ is in no way invalidated by recognition of the Archaeobacteria and Eubacteria. This fundamental divergence, and their even more fundamental similarity in prokaryotic cell structure, can be most usefully represented by ranking Eubacteria and Archaeobacteria as kingdoms within the superkingdom Prokaryota¹², rather than as subkingdoms^{1,3}.

The differences between halobacteria and photosynthetic eubacteria (all negibacteria) are so great that to place them in a 'photocyte' group¹⁶ that excludes other archaeobacteria is taxonomically meaningless. The affinities of the archaeobacteria lie instead with Gram-positive eubacteria

on the one hand and eukaryotes on the other¹². The phylogeny shown in the accompanying figure is consistent with cell structure¹², with the 16S rRNA tree (if rooted where eubacterial phyla diverge)⁸, and with the fossil evidence that eubacteria evolved 2,500 Myr before eukaryotes¹⁷. Lake's opinion^{6,7} that eubacteria have a "fast clock" is counter to the fossil record which suggests they are the most conservative of the three groups, several times more so than eukaryotes.

Postulating a 'fast clock' or a 'slow clock' for a whole group does not explain the close bunching within, compared with the wide separation between, the three major lineages on the rRNA tree⁸. This pattern can be produced only by a temporary phase of accelerated rRNA sequence change during the divergence of the three lineages (on my tree at the time indicated by the asterisk, not separately in three lineages diverging from the progenote as postulated by Woese).

Because it confuses phylogeny and classification, and is objectionable to eukaryote biologists, the misleading phrase 'three primary kingdoms' should be eschewed and replaced by the accurate 'three major lineages' when referring to the eubacterial, archaeobacterial and eukaryo-

tic branches of the tree of life. As there are two kingdoms of prokaryotes, and with the separation of the Chromista and Protozoa, five monophyletic eukaryotic kingdoms, we should speak of 7 not 3 kingdoms.

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How many reactor accidents?

SIR—It does not take advanced statistical calculations to conclude that there is a disturbingly high probability of another serious nuclear reactor accident in the next ten years, given that there have been two so far amongst 374 reactors with a total of 4,000 reactor-years experience. In their letter, Islam and Lindgren¹ are undoubtedly right to conclude from these data that the true probability is irreconcilable with the probability apparently claimed by reactor designers on the basis of 'technical risk assessment'. Their statistical arguments, however, are characterized by a failure to distinguish between probability and likelihood, which has led them to make unwarranted statements.

Assuming a Poisson process with parameter r (reactor-years⁻¹) they correctly find the probability of two accidents in $T = 4,000$ reactor-years to be $\frac{1}{2}(rT)^2 e^{-rT}$. But they then treat this as an unnormalized probability distribution with parameter r , writing "If we want this to be a probability density, we have to normalize it". But one cannot turn any mathematical function into a probability distribution just by making it integrate to unity, and the function in question is a likelihood function for r , not a probability distribution, and cannot be integrated².

By treating the likelihood function as though it were a probability distribution, the authors have unwittingly adopted a Bayesian approach with implicit prior

probability distribution dr for r . But even if one wished to admit the Bayesian approach here, no Bayesian would use this prior for a non-negative parameter since its integral obviously diverges and it distorts any analysis in favour of the larger values of r . The commonest Bayesian recommendation³ is to adopt the arbitrary prior distribution $(1/r)dr$, which would have the effect of shifting the graph of the authors' Fig. 1 to the left, placing its maximum at $r = 0.00025$ rather than 0.0005.

Islam and Lindgren then conclude from an integration of their probability distribution between two values of r that "This [the value 0.21] indicates that the probability that one significant accident will occur between 1,667 and 2,500 reactor years is 21%", a strange statement which presumably rests on an erroneous inter-

pretation of their probability distribution for r (the caption to their Fig. 1 suggests this explanation).

The authors next compute the probability of at least one accident in t reactor-years, integrating out the unknown r in accordance with the Bayesian posterior probability distribution they have derived for it on the basis of the prior distribution dr and the two observations. They conclude "The probability that a reactor accident could happen during the next decade is 86%" (another strange statement since the probability that an accident could happen is manifestly 1; they mean will).

Aside, however, from these detailed criticisms, there remains the major issue of treating a likelihood function as though it were a probability distribution. Avoiding this issue, the analysis might proceed as follows.

As stated above, $\frac{1}{2}(rT)^2 e^{-rT}$ is the likelihood function for the unknown parameter r in the light of two accidents in $T = 4,000$ reactor-years, and, as the authors say, the probability of at least one accident in t reactor-years is $P = 1 - e^{-rt}$, and r is unknown. But rather than adopt a probability distribution for r we should use the above likelihood function for it and derive the corresponding likelihood for the unknown P by simple substitution: $r = -(1/t)\ln(1-P)$. So the likelihood for P is;

$$L(P) = \frac{1}{2}[-(T/t)\ln(1-P)]^2 \exp[(T/t)\ln(1-P)]$$

It is customary to work with the standardized log-likelihood, or *support*⁴, which is;

$$S(P) = 2 \ln[-(T/t)\ln(1-P)] + (T/t)\ln(1-P) + 2 - 2 \ln 2$$

(support is standardized by adding a constant so that the support function is zero at its maximum).

Figure 1 shows the support function for P with $T = 4,000$ and $t = 10 \times 374$ reactor-years. The maximum-likelihood value for the probability of at least one explosion in ten years is seen to be 0.85 but, as is entirely to be expected with only two observations, there is great uncertainty about this value, the 2-unit support limits being 0.26 and 1.00. Islam and Lindgren's Bayesian analysis, by confounding likelihoods and probabilities, completely obscures this uncertainty, and consequently confers spurious scientific accuracy on what can only be an educated guess. All we can really say is that the probability of an accident in the next ten years is unlikely to be less than a quarter — a sufficiently alarming prospect not to detract from the main point they were making.

It may be tiresome to have to maintain these distinctions between likelihood and probability, but they are of especial importance when dealing with sparse data and infrequent events. Physicists usually use statistical theory in connection with

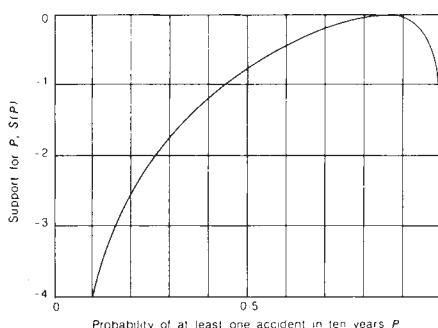


Fig. 1 The support for various values of the probability of at least one reactor accident in ten years, given two accidents in 4,000 reactor-years so far and the continued use of 374 reactors worldwide.

large samples, where the finer points of statistical inference are relatively unimportant, but biologists often have to argue from quite small samples. It is no accident that the greatest impetus for the refinement of the logic of statistical inference has come from the biological sciences, and especially from human genetics, where the paucity of the data is matched only by the importance of the inferences. Nuclear accidents fall into the same category.

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Stunning whales

SIR—M.A. Taylor's comments on prey echolocation and sonic squid-stunning by whales (*Nature* **323**, 298, 1986) contain an incorrect discussion of the relation between the maximum detection distance by the whale (D) and the maximum distance at which potential prey can detect the whale's echolocating emissions (d). If for simplicity we neglect absorption, refraction, scattering by extraneous bodies, inhomogeneities, anisotropies, near-field and frequency-dependent effects, then the outward power-flux at distance r is $W/4\pi r^2$, and the returning power-flux experienced by the whale is $W\sigma/16\pi^2 r^4$, where W is the emitted power and σ is the backscattering cross-section of the prey. If the minimum flux detectable by the whale is S and by the prey is s , we may find D and d from the equations $S = W\sigma/16.235^2 D^4$ and $s = W/4\pi d^2$. The ratio d/D is $(WS/\sigma s)^{1/4}$. This expression can be made more illuminating by normalizing to prey-length, L , letting $\sigma = \alpha L^2$ and $D = \beta L$, yielding $d/D = 2\beta(S/\alpha s)^{1/4}$. For any useful sonar system, the maximum prey detection range will be several prey-lengths ($\beta > 1$), and typically $\alpha < 1$.

Thus, for roughly equal hearing sensitivity, d/D will be greater than unity, and the prey will be able to take evasive action before detection by the whale. The ratio d/D is not 2, as indicated by Taylor's Fig. 1. This does not affect his fascinating discussion, but the relation given here permits additional variations of strategy in Taylor's "evolutionary arms race".

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Body temperature and the specific heat of water

SIR—John Paul attempts to explain why the normal body temperature of homoiotherms is approximately 36 °C by its proximity to the temperature of minimum

specific heat of water¹. He states: "An organism functioning at this temperature will find it necessary to generate or dissipate the minimum amount of heat energy in order to maintain its temperature constant". But the rate of heat loss (and therefore rate of heat generation with which it is balanced) is equal to the temperature differential between body and environment times the heat transfer coefficient ("conductance"). This is independent of heat capacity, or heat content (specific heat times mass times temperature change). In fact, temperature would be maintained more easily if the specific heat and heat content were greater — that is, for a given amount of heat loss, the temperature decrease would be less.

Perhaps a better reason for maintaining body temperature considerably higher than the average ambient temperature is that this ensures that excess heat produced by the high metabolism of terrestrial mammals and birds can be dissipated by conductive, convective and radiative means which require no evaporative water loss².

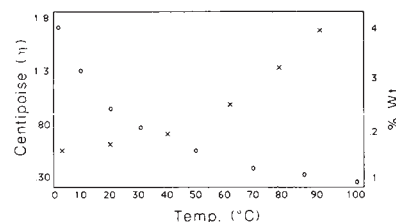
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SIR—We were intrigued by the proposal¹ that the nearly constant body temperature (around 36 °C) of many homoiothermic animals can be explained by the fact that the specific heat of water passes through a minimum around this temperature. The explanation offered was that "an organism functioning at this temperature will find it necessary to generate or dissipate the minimum amount of heat energy in order to maintain its temperature constant. From the point of view of the organism's energy economy this temperature is clearly the most efficient at which to function".

This explanation is appealing but it cannot be correct. The amount of heat required to maintain a given temperature is, of course, exactly equal to the amount of heat lost to the environment; this depends on many factors, such as the temperature difference, surface area and thermal conductivity of a body, but it is independent of its specific heat. Moreover, the smaller the specific heat, the larger will be the fluctuations in the temperature of the body, so that the problem of maintaining a constant body temperature becomes all the more difficult. Finally, the specific heat minimum at about 36 °C applies to pure water. Aqueous solutions generally shown no minimum in the specific heat, which usually decreases monotonically as the temperature drops and is appreciably lower than that of pure water in the re-



Crosses, solubility; circles, viscosity.

levant temperature region. The specific heat of normal blood plasma is about 6 per cent lower, that of haematopoietic cells about 11 per cent lower².

What then is the explanation? We do not know, but most likely, a temperature of around 36 °C, if it is the product of natural selection at all, is selected because it corresponds to an optimal mix of properties. We consider here only two, viscosity and 'hydrophobic' effects.

The rates of many biological processes are limited by diffusion. As temperature rises, long-range structures in water are disrupted, the viscosity decreases, and the rates of diffusion-limited processes become faster. This factor by itself would appear to favour higher body temperatures. But disruption of the long-range structures in water will also lead to changes in hydrophobic effects, which are important in many vital processes, such as substrate binding, protein folding and bilayer membrane formation.

The accompanying figure shows the viscosity of water and, as a measure of hydrophobic forces, the solubility of benzene in water³, both as a function of temperature. By inspection, a temperature of around 36 °C seems to be a reasonable compromise; high enough to give a low viscosity, low enough that hydrophobic molecules do not dissolve too easily.

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Reproductive failure in common seals

THE table shown below was omitted from the letter by P.J.H. Reijnders on page 456.

Table 1 Number of participating, ovulating and pregnant seals in both experimental groups, during the season 1983-84

Group	1	2
No. of females	12	12
No. ovulating	12	12
No. pregnant	4	10

What is supersymmetry?

Michael Atiyah

Supersymmetry and its Applications: Superstrings, Anomalies and Supergravity.

Edited by G.W. Gibbons, S.W. Hawking and P.K. Townsend.

Cambridge University Press: 1986. Pp.481. £27.50, \$49.50.

PHYSICISTS are an ambitious lot. Their aim is to understand the Universe and what makes it tick. What is matter? What are the forces that act on it? How did it start and where will it end? These, no less, are the monumental questions that theoretical physicists attempt to answer. So far, in historical terms, their track record in answering these questions is quite impressive. The two great triumphs of earlier centuries, Newton's theory of gravitation and Maxwell's theory of electromagnetism, remain outstanding examples of what a physical theory should be. Universal in application, and capable of simple and rigorous formulation in mathematical terms, they provide a firm basis from which to attack complex and realistic problems.

At the core of both theories are the notions of "particles" and "fields", that is of matter and force. Particles produce fields and fields act on particles in a perpetual duet. As physicists have delved further into the nature of this interaction, the theories that have emerged have become more and more complex, both conceptually and mathematically. Einstein altered our view of the separateness of space and time, and his theory of general relativity is much more sophisticated than Newton's, although equally rigorous in its mathematical formulation. Quantum mechanics involved an even greater revolution and its foundations are much less clear. "Elementary particles" have been emerging from the laboratories in ever-increasing numbers, and theories to explain them have been just as numerous.

In all this excitement and turmoil two main problems have always stood out. What are the ultimate "elementary particles"? And how does one reconcile general relativity with quantum mechanics? The first of these is closely tied to experimental discoveries and alters with each new generation of accelerators, while the second is a purely intellectual or mathematical problem because gravitation and quantum mechanics operate at quite different scales.

Quantum field theory aims to provide a theory of elementary particles consistent with quantum mechanics and based on a sophisticated unification of the notions of particle and field. The details of the theory may vary depending on how many kinds of particle one postulates. In fact, it is extremely difficult to construct any mathematically

consistent quantum field theory. Many theories are plagued with "ultraviolet divergences" or "anomalies" and the mathematical study of these problems has become quite an industry.

Given all the difficulties, it is not hard to understand why, in the past couple of years, there has been such widespread enthusiasm amongst theoretical physicists for what are called "superstring" theories. These theories appear to offer the first realistic chance of a consistent account of nature which combines quantum field theory and general relativity. As the term suggests, these new theories are a combination of two different ideas, namely strings and supersymmetry, both of which have been investigated separately for some time.

In one form or another, symmetry has always played a prominent part in physics. Essentially, the requirement that a physical law be independent of the particular location or orientation in space or time means that the relevant theory has to admit the appropriate symmetries of space-time. Beyond this physicists have found that elementary particles tend to come in families, and the best explanation has again been couched in terms of symmetries, not of space-time but of some "internal" space.

Particles in quantum mechanics come in two different species, depending on their "spin". There are fermions, which have half-integral spin and obey the Pauli exclusion principle, and bosons which have integral spin and have symmetric wavefunctions. Physically they are fundamentally different but supersymmetry ignores this difference and puts them on an equal footing. Roughly speaking, a supersymmetric theory should allow the interchange of fermions and bosons. Despite its physical implausibility supersymmetry has many theoretical attractions, and the mere requirement that a theory be supersymmetric is so strong that it greatly limits the number of possible candidates. For these reasons it has been extensively studied.

String theory starts from the idea that the idealized picture of a point-particle should be replaced by that of a string. This also has been studied, off and on, for many years with varying degrees of enthusiasm and optimism. The subject really hit the headlines however when, in its supersymmetric form, it was discovered by John

Schwarz of Caltech and Michael Green of Queen Mary College that a consistent anomaly-free theory could be constructed. This would in principle give a unified theory of elementary particles, incorporating gravity and quantum mechanics. Since then, there has been intense activity developing the several variants of the superstring theory and examining their consequences.

In 1985 a workshop in Cambridge, UK, was essentially devoted to these latest developments, and this volume records the various contributions. Many of the leading experts were present, and their talks give an authoritative view of the situation. While the material is highly technical, a few talks were addressed to the uninitiated, notably the introduction to strings by Edward Witten of Princeton.

Although superstring theories hold great promise and have generated renewed optimism about unified field theories, they are still very mysterious. The foundations are not at all clear, and surprises are probably in store for us. At the mathematical level, strings are difficult to understand, but it is already clear that they will involve many new techniques and ideas. It should be emphasized that the unification of forces involved in superstring theory takes place way beyond the realms of direct experimental verification. Thus the main tests of the theory are those of internal mathematical consistency. However, these are extremely severe. In fact physicists would like to believe that there is only one possible consistent solution. All they have to do is find it!

Perhaps the final word should be given to Witten, one of the high-priests of the subject, who, having explained the peculiar mathematics of superstrings, goes on to say:

But the fact that these things work, that these seemingly bizarre rules give ways of computing things in quantum gravity, giving sensible results and finite answers and leading in many different directions to all kinds of beautiful areas in mathematics, is a very deep mystery — probably one of the deepest which has ever been encountered in physics. It is unlikely that a proper understanding of this mystery will be found either soon or simply. But it will be worth the wait. □

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• Accounts of supersymmetry and associated topics are now reaching textbooks for advanced undergraduates and postgraduates. Four such books have appeared over the past few months and will be reviewed in a future issue of *Nature*: *Introduction to Supersymmetry* by P.G.O. Freund; *Introduction to Supersymmetry and Supergravity* by P. West; *Supersymmetry, Superfields and Supergravity: An Introduction* by P.P. Srivastava; and *Supersymmetry: A Decade of Development* edited by P. West.

nonlocality and its apparent conflict with relativity may look very different from a deterministic and an indeterministic point of view.

David Deutsch explains and defends his own version of the Everett many-universes interpretation, that reality "splits" every time we take a measurement. His claim that the existence of the resulting "parallel" universes could be demonstrated experimentally is interesting but undoubtedly controversial. John Wheeler in his interview explains why he has now abandoned support for the Everett approach — "too much metaphysical baggage", as he puts it. He now leans to the view that the existence of conscious observers is crucial to quantum observation, a view definitely not to be ascribed to Bohr. Indeed it is fair to say that the whole language of observers and observables that clutters up books on quantum mechanics is an unfortunate legacy of a long-outmoded positivist attitude to the nature of science, but which tends regrettably to invite the casual reader to incorporate an ineliminable subjectivity into the understanding of quantum mechanics.

John Taylor attempts to defend what he calls the statistical interpretation, that quantum mechanics is a theory about ensembles of identically prepared systems, and has nothing to say about the single system. It is quite true that the nonlocality difficulties do not arise in terms of statistics. But Taylor's discussion of the Einstein-Podolsky-Rosen experiment is very confused, leading one to believe that mirror-image correlations do not exist in the statistics of measurement results on the same spin-component of the two particles. For example, Taylor says that "We can't say in any particular case what that spin of the far away particle is from the measurement nearby". This is potentially misleading, because it might be taken to imply that we cannot predict what the result of a subsequent far-away measurement would reveal, and that is just wrong. Presumably Taylor means that we cannot say what the spin of the far-away particle was *before* the measurement on the nearby particle. This is much closer to Bohr's position than what most people would regard as the statistical interpretation.

In all, this book is a useful introduction to a variety of ideas and approaches about understanding quantum mechanics. It shows that there is no longer a regimented uniformity of view even among "respectable" physicists. That is a healthy sign: dogmatic metaphysical strait-jackets are best consigned to the scientific lumber-room. □

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Captured in clay — 15,000-year-old models of bison from Le Tuc d'Audoubert cave in the French Pyrenees; the animal in the foreground is 60 cm long. The picture is reproduced from Dark Caves, Bright Visions: Life in Ice Age Europe, by Randall White, an account in words and pictures of the accomplishments of the inhabitants of Europe during the Upper Palaeolithic (35,000–12,000 years BP). The book is based on an exhibition at the American Museum of Natural History, New York, and is published by W. W. Norton. Price is \$35.

Change in the field

Subir K. Banerjee

Environmental Magnetism. By Roy Thompson and Frank Oldfield. Allen & Unwin: 1986. Pp. 227. £35, \$50.

THE TITLE of Thompson and Oldfield's book may lead one to think that it deals with the magnetic field and its fluctuations in the human environment. This, however, is not the case. The authors' respective backgrounds are in geophysics and geography, and over the past decade or so they have worked together on the magnetism of terrestrial materials — such as soil, and sediments deposited in lakes, peat bogs and oceans — with the purpose of reconstructing environmental and climatic changes. In spite of the complexities of natural magnetic minerals, the myriad ratios in which two or more such minerals can be present and the simplicity of the magnetic apparatus used, many of the stories told and the examples given constitute brilliant successes in scientific detective work.

Because of the many examples listed, the book will probably be of most use to non-magnetists interested in palaeoenvironmental change. It is well-structured, in that the first six chapters cover the basics of magnetism, magnetic minerals and the behaviour of the geomagnetic field, while the remainder deal with the applications to nature. The diagrams are many and of excellent quality, although a large number

of them suffer from lack of acknowledgment of the source so that the reader will mistakenly think that they are either the results of the authors' own work or "given truths" in rock magnetism. For example, in Fig. 4.9 the authors purport to show the grain-size dependence of the ratio of susceptibility to saturation remanence for magnetite, but it is not made clear that these are the results of a thought experiment only. Similarly, on p. 56 a non-magnetist not familiar with the literature may interpret the authors' hypothetical model of frequency dependence of susceptibility of very small magnetite grains to be proven, while a thorough test of such a model has yet to be made.

It is important to emphasize that nature's cooperation in sometimes providing a simple mixture of one strongly magnetic and one weakly magnetic mineral (for example, magnetite and haematite) has been most helpful to the authors in solving their magnetoforensic problems. In discussing one particular topic, the magnetism of lake sediments, Thompson and Oldfield themselves point out that "further progress, as distinct from the pragmatic application of the approaches illustrated above to new sites will depend on ... closer and more quantitative specification..." (p. 123). I believe that the statement applies to most of the applications of magnetism to the environment, as described in this book. □

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Other sides of the astronomer

Robert S. Westman

Nicholas Copernicus: Complete Works. Vol. III Minor Works. Edited by Paul Czartoryski. Translation from the Polish edition and commentary by Edward Rosen with the assistance of Erna Hilfstein. *Macmillan, London: 1986. Pp.380. £60.*

FEW scientists and general readers are aware that Copernicus's first published work (1509) was a translation from Greek into Latin of a book entitled *Ethical, Rustic, and Love Letters* by an obscure ninth-century Byzantine writer named Theophylactus Simocatta. "Your natural beauty has faded and your good looks are approaching [the stage of] wrinkles", so opens letter No. 3. "But you try to give the impression of truth when you deceive your lovers with artificial cosmetics. Harken to time, you hag, for in the autumn meadows are not the place for flowers." Etc. It is good fun and a fine way for a young humanist to sharpen his Greek. And, because Copernicus's Greek dictionary has survived, it is possible to see just how good his Greek was given the lexicographical tools at his disposal. Because every letter in his dictionary began with a capital letter, he could never be certain that he was considering a proper or a common noun. In letter 31, the peacock is described by Simocatta as "the Persian bird" who acquires "the arrogance of the Persians", but Copernicus produced instead "an active bird" that took on the "splendor of the energetic birds" (p. 8).

In 1517, Copernicus, a canon or administrative official of the Warmia chapter of the Church, composed a Latin treatise on the urgent matter of the debasement of coinage; two years later, at the behest of the West Prussian Estates, he prepared a German version that differs in certain respects from the first. "It is not the least advisable to introduce a new, good coinage", wrote Canon Copernicus, "while an old, debased coinage remains in circulation. How much worse was this mistake, while an old, better coinage remained in circulation, to introduce a new debased coinage, which not only spoiled the old coinage but, so to say, swept it away!" (p. 187).

The passages cited above are but two examples of the rich feast to be found among the "minor works" of Nicholas Copernicus, a figure otherwise known in the history of science for daring to propose that the Earth and the other "elements" beneath the Moon revolve annually around a motionless Sun. To be sure,

astronomical writings are not missing from this volume: the *Commentariolus*, Copernicus's first formulation of the heliocentric hypothesis, probably written sometime between 1508 and 1514, and not published in Copernicus's lifetime; the *Letter Against Werner* (1524), which attacks Johann Werner's treatise on the motion of the sphere of the fixed stars but deferring to another occasion the author's own views on whether that sphere moves or not. In addition, one finds translations of all 17 extant letters written by Copernicus as well as a group of administrative documents. Copernicus had studied medicine at Padua; in one of his books, one finds a prescription for pills that promises to do the following:

harmlessly eliminate whatever is excessive... postpone gray hair, which comes from corrupt humors... repress catarrh, stop a cough, relieve the congestion... alleviate gas in the stomach... sharpen the wits, strengthen and invigorate the nerves, preserve the teeth from decay... induce sleep... and purge gently [p.301].

Vast talents and international energies went into the preparation of this volume. Professor Paul Czartoryski, general editor of the *Complete Works*, deserves the highest praise for the difficult task of coordinating the Polish and American research teams. His job was not an easy one. Although the late Edward Rosen, assisted by Erna Hilfstein, produced the English translation, their work built significantly but with thin acknowledgement upon the Polish contributions. Few of the scholars mentioned in Czartoryski's detailed introduction, such as Jerzy Dobrzycki and Jerzy Drewnowski, are referred to in Rosen's notes; indeed, scholars will need to consult the Polish edition for material arbitrarily excluded by Rosen and Hilfstein.

On the other hand, a veritable firestorm of polemic fills Rosen's footnotes to the *Commentariolus* (pp. 91-126) where Noel Swerdlow (baptized as "Sw" in order to eliminate him from the Citation Index!) is roasted to a crisp for having had the temerity to translate anew in 1973 Rosen's supposedly canonical 1939 version of that same work. Rosen has now adopted some of Sw's language in this new translation—without acknowledgement. The principle of ignoring what you take from others while castigating them for their minor peccadilloes, real or imagined, seems here to have been raised to the status of a major editorial axiom. Copernican specialists will easily spot these off-the-text dog fights; general readers will probably miss them (without loss). Everyone will benefit from what is left over: a much richer picture of Copernicus as a Renaissance humanist, church astronomer and man of practical action. □

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US Supreme Court to review Louisiana appeal

Thomas H. Jukes

An alliance of scientists and educational and religious leaders has converged to defend evolution from determined attack by the creationist lobby.

THE appeal in the case *Edwards et al.* appellants [Edwin W. Edwards is Governor of Louisiana] versus *Aguillard et al.*, appellees, will be heard by the US Supreme Court next week (10 December). The case pits creationism against evolution. The action originated in a 1985 opinion of the US Court of Appeals, affirming the unconstitutionality of an act passed in Louisiana "Balanced Treatment for Creation-Science and Evolution-Science in Public School Instruction, Requiring the Teaching of Creation Science in Louisiana Public Schools Whenever Evolution is Taught".

The act requires that textbooks, library materials, and educational programmes include balanced coverage of both 'creation science' and evolution; that both evolution and 'creation science' be taught as theories rather than as proven scientific facts; that each local school board develop and provide to each public school teacher a curriculum guide on the presentation of 'creation science', and that the governor designate a panel of seven 'creation scientists' to assist local school boards in the development of these curricula. The act argues that "creation science consists of scientific evidence and not religious concepts" and therefore should be taught in science classes. The act states that its purpose was "For protecting academic freedom." However, the US Court of Appeals ruled that "the principle of academic freedom abjures state interference with curriculum or theory as antithetical to the search for truth. The balanced treatment act is contrary to the very concept it avows . . ."

Religiosity

The strategy used by creationists in writing the Louisiana act was developed as a response to their 1982 court defeat in Arkansas, where "the inescapable religiosity" of creationism had been exposed by Judge Overton. In the Louisiana act they kept mum about the religious nature of creationism and pretended that a viable 'creation science' existed.

The Governor, in his official capacity, together with the Attorney General and State Superintendent of Education of

Louisiana, are appealing against the decision of the US Court of Appeals of the State. The legislature passed the act by a large majority, and it appears to have a strong popular appeal in Louisiana. The appellants are heartened because the Appeals Court ruled on a majority of only one: seven judges dissented.

The US Supreme Court procedure is that the appeal will be argued by lawyers for the appellant and appellees. Permission has been granted for other interested parties to file *amicus curiae* briefs. These will be added to the record to be read by the Court, but the lawyers who filed the briefs will not be allowed to present oral arguments on 10 December.

FASEB's brief

The Supreme Court has received 16 *amicus curiae* briefs, most of them in support of the appellees. As far as I know, the scientific and educational communities have never previously joined together on a legal issue to a comparable extent. One brief for the appellees is joined by the Federation of American Societies for Experimental Biology with 28,000 elected members, nearly all with doctoral degrees. Another brief was filed by 72 Nobel laureates and 17 state academies of science. Probably this arousal of activism has come about because creationism threatens other natural sciences, such as astronomy, astrophysics and geology, in addition to biology. A third brief for the appellees was filed by four clergymen.

A typical statement by creationists is that they are "fully committed to the inerrancy of Scripture", as well as to "the strict creationist young Earth position taught in Scripture . . ." This places the date of creation as about 10,000 years ago, which accounts for opposition by creationists towards any of the natural sciences that state that the Earth and the Universe are thousands of years old. Creationists reject radiometric dating, by claiming that isotopic changes do not occur at uniform rates. Similarly contrived arguments are used by creationists against any and all scientific findings that are in contradiction with a literal interpretation of the King James version of the Holy Bible.

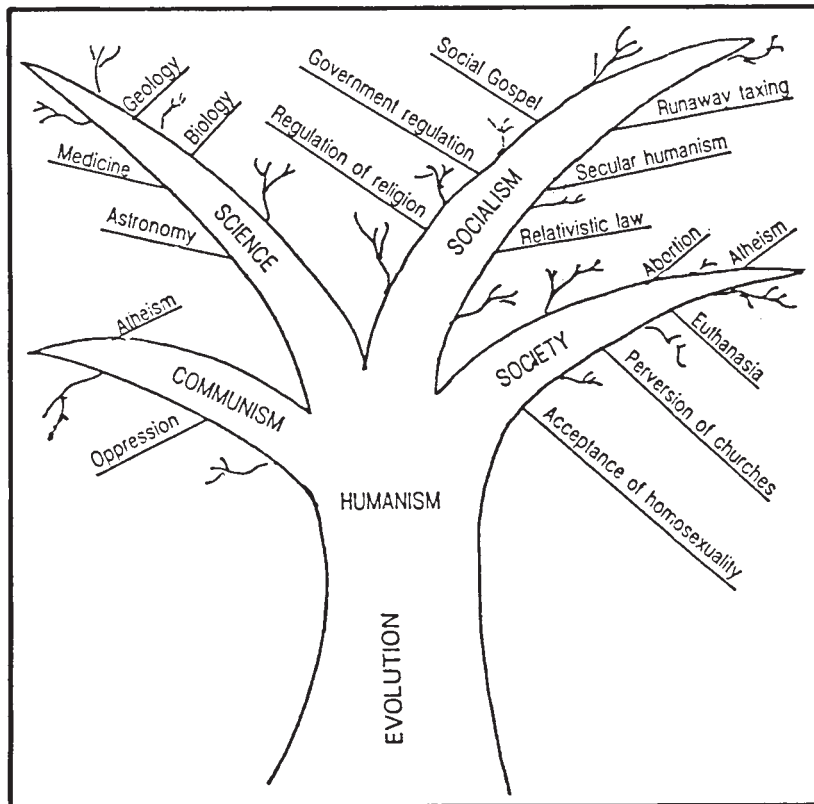
An irony is that the present case depends on legalities surrounding the establishment clause of the United States Constitution rather than on the validity of evolution. Only one brief, that of the US National Academy of Sciences, says anything descriptive about evolution, in a discussion of divergence of cytochromes *c* in terms of the molecular evolutionary clock. News of the current studies of proteins and DNA that reveal relationships of all living organisms with each other have not yet reached most people, and, of course, the knowledge has been deliberately excluded from most public school science textbooks by the successful efforts of creationists. This exclusion is a victory for obscurantism. It is therefore strange to read in *Nature* articles and letters affirming that creationism is a rather interesting sociological phenomenon, and one that should be treated sympathetically^{1,2}.

Four of the briefs are on behalf of the appellants, that is, for creationism, by the Christian Legal Society, the Rabbinical Alliance of America (joined by Congressman William E. Dannemeyer, who is well known for his opposition to the pasteurization of milk), the Catholic League for Religious and Civil Rights (anti-abortion) and, as mentioned below, Concerned Women for America.

The other 12 briefs are for the appellees, and usually emphasize the constitutional separation of church and state which the Louisiana Act would violate, also the attack on academic freedom presented by the act, and the threat to the teaching of other secular subjects, in addition to evolution, that is posed.

"Anti-Christian"

In view of the pending Supreme Court case, a ruling by US District Court Judge Thomas G. Hull in Greenville, Tennessee on 25 October, 1986, takes on added significance. He ordered public schools in Hawkins County, Tennessee, to excuse fundamentalist children from attending classes where they would be exposed to books that their parents say promote themes which they regard as "anti-Christian." The parents objected to stories in the Holt-Rinehart-Winston books



"Evolution is the taproot which is feeding the oppressive, murderous and infidel directions we see gaining acceptance around us . . ." says Paul Bartz of Bible Science Newsletter.

about "Cinderella" and "Macbeth," because of their mention of magic, to *The Wizard of Oz*, because it portrayed a witch as good, and *The Diary of Anne Frank* because it suggested all religions are equal. Ominously, another objection was to stories about dinosaurs if the creatures were said to be older than the Biblical account of the beginning of the world, presumably 10,000 years ago. Judge Hull's decision included a statement that "the plaintiffs believe that after reading the entire Holt series, a child might adopt the views of a feminist, a humanist, a pacifist, an anti-Christian, a vegetarian, or an advocate of "one world government". He found that the plaintiff's objections were sincerely held religious convictions, thus apparently conceding that objections to almost anything should be upheld if under the guise of religious convictions. The leading complainant also objected to reading about catholicism, which she said "could produce changes in my children's way of thinking — they could become confused".

It is of added significance that Michael Farris, the plaintiffs' chief attorney, is the author of the *amicus curiae* brief of the Concerned Women for America in the Supreme Court case. In it, he argues that "the Louisiana law has the clear secular purpose of promoting academic freedom and of insuring that students learn the scientific evidence of both evolution and creation". This fallacious argument is

based on the erroneous presumption that there is scientific evidence for creation, which evidence actually rests on literal acceptance of the Book of Genesis. It is noteworthy that the Concerned Women for America, in one court action, object to their children reading about Cinderella, and in a second court action would foist creationist travesties of science on all children.

Judge Hull also stated "This opinion shall not be interpreted to require the school system to make this option available to any other person or to these plaintiffs for any other subject." Does this statement represent a disturbing challenge to the doctrine of *Stare decisis e non modere quieta*, which could thus be used against previous decisions that permit the teaching of evolution?

Another component that must be weighed in a Supreme Court case is the presence of William Rehnquist in his newly appointed role as Chief Justice. Given the impact of the recent US Court decision in *Greenville*, what can we expect from him? It should be noted that the US District Court first threw out the case in December 1983, following which the parents appealed to the United States Court of Appeals in Cincinnati. This court sent the case back to the lower court, holding that the parents' complaints deserved the hearing of a trial, with the result that the parents won. Will the Supreme Court react to this precedent?

Taxation

The fantastic accusations against evolution by the creationists are seemingly endless. The figure shown above accompanied an article written in 1983 by Paul A. Bartz, the executive editor of *Bible Science Newsletter*. The article included the sentence "Evolution is the taproot which is feeding the oppressive, murderous and infidel directions we see gaining acceptance around us . . ." Mr Bartz builds a powerful case for his opposition to evolution by alleging that it leads to "runaway taxing." In a sense, this is quite true, in that runaway taxing did not exist before the appearance of vertebrates.

It is more likely, however, that by "evolution" Bartz refers to the teaching of evolution rather than to its existence. A sample of creationist "teaching material" for young children¹ contains a cartoon for each letter of the alphabet. The letter *A* shows Adam and Eve surrounded by dinosaurs; *B* for Bible and brachiosaurus has Adam at his first job — naming the brachiosaurus. *F* says that fossils were formed by the sudden inundation of the Biblical Flood, but "normally, when an animal dies, no fossil is formed". We are assured that "God put dinosaurs on the Ark". A chilling cartoon on *U* (for "unchanged") implies that, just as the mockers of Noah were drowned, those who today jeer at church-goers will be destroyed by the vengeful Deity.

Evolution started in Darwin, and grew with genetics. The mechanism of inheritance had to provide for changes occurring during long periods of time. Information in the genes is used every time a living organism develops from a fertilized egg cell; this event, because it happens so quickly, is far more remarkable than evolution. The pervasive influence of biochemistry grew steadily; biochemists showed the universal nature of many metabolic pathways and showed that all living organisms had coenzymes containing the same remarkable molecules. Specific procedures have firmly established evolution at the centre of the biological sciences, aided by population genetics, plate tectonics and computer sciences. "Gaps in the fossil record" are irrelevant in the face of molecular evidence. All lines of scientific inquiry show that the great apes are our closest relatives. We are sorry that the creationists do not approve of their cousins, but, regardless of the Establishment Clause, this is the way it happened. □

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A thyromimetic that decreases plasma cholesterol levels without increasing cardiac activity

A. H. Underwood, J. C. Emmett, D. Ellis, S. B. Flynn, P. D. Leeson, G. M. Benson, R. Novelli, N. J. Pearce & V. P. Shah

Smith Kline and French Research Limited, The Frythe, Welwyn, Hertfordshire AL6 9AR, UK

A number of agents that mimic the ability of the thyroid hormone, T_3 , to decrease plasma cholesterol levels are described; one is as effective as T_3 at reducing cholesterol levels and stimulating liver function, but has very little effect on cardiac function and is thus less likely to be toxic. The agent may be useful in the treatment of atherosclerosis.

CHOLESTEROL concentrations are increased in plasma in hypothyroidism and diminished in hyperthyroidism; treatment of thyroid disease normalizes plasma cholesterol¹. In man², and animal models^{3,4} with normal thyroid function, relatively low doses of thyroid hormones reduce circulating cholesterol. But 3,3',5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) cannot be used therapeutically to treat hypercholesterolaemia because of the adverse consequences of some of their other actions, notably on the heart. The cardiac effects could either be a direct consequence of the interaction of the hormones with their cardiac receptors, or a result of the characteristic increase in metabolic rate leading, indirectly, to increased cardiac output. Several groups of workers have attempted to design analogues that retain hypocholesterolaemic activity but lack cardiac and metabolic effects. However, despite claimed selectivity in animals for certain compounds^{3,5}, all agents that mimic the thyroid hormones (thyromimetics)^{6,7}, including D- T_4 (ref. 7), have shown unacceptable cardiac side effects when tested in man.

Thyroid hormones act on the liver in several ways that could increase cholesterol elimination. These include induction of low-density lipoprotein (LDL) receptors⁸, lecithin-cholesterol acyl transferase^{2,9}, hepatic lipase^{2,10} and lysosomal acid cholesterol esterase¹¹. Thus an agent that mimics the thyroid hormones in their hepatic but not their cardiac activity could be a valuable agent for treating hypercholesterolaemia. Here we describe a new class of selective thyromimetics. The most potent of these, SK&F L-94901, after chronic administration, is as active as T_3 on the liver but has about 0.1% of the activity of T_3 on the heart. We also show that this compound is a very potent hypocholesterolaemic agent with only a small effect on metabolic rate.

Experimental approach

The primary assay used was the induction of the enzyme GPDH (mitochondrial cytochrome *c* 3-phosphoglycerate oxidoreductase) in the heart and liver of a hypothyroid rat. Both natural and synthetic thyromimetics caused large (3–6-fold), easily quantified increases in cardiac and hepatic GPDH 48 hours after the administration of a single dose to hypothyroid rats (A.H.U. and D.E., unpublished data). The assay gave similar results to ones based on changes in whole body or tissue oxygen consumption, and is thought of as an excellent measure of the effects of thyromimetics on specific organs¹². Secondary screening involved chronic administration, usually oral, of selected compounds to either hypothyroid rats or rats with normal thyroid function, to determine hypocholesterolaemic activity and effects on whole body oxygen consumption (WBOC) and thyroid function.

We have used measurements of the affinities of novel compounds for the thyroid hormone receptor of isolated cardiac

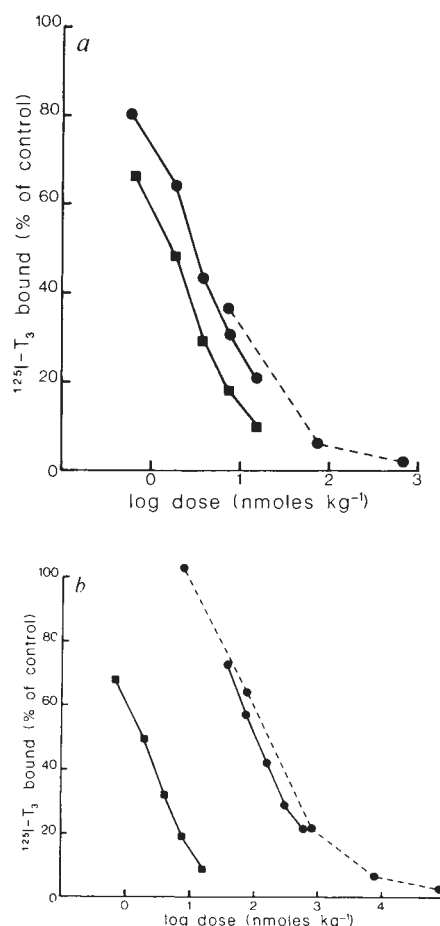


Fig. 1 Competition of T_3 and SK&F L-94901 with ^{125}I - T_3 for occupation of nuclear receptors *in vivo*. *a*, hepatic nuclei; *b*, cardiac nuclei; ■, unlabelled T_3 ; ●, SK&F L-94901.

Methods. Rats were rendered hypothyroid by providing them with drinking water containing 0.3 g l^{-1} of the antithyroid agent 2-mercapto-1-methylimidazole (MTI) for at least 4 weeks. They were then given intravenous injections of ^{125}I - T_3 and various doses of unlabelled T_3 or SK&F L-94901. One hour later hepatic and cardiac nuclei were isolated²² and the ^{125}I and DNA²³ content determined. Nuclear ^{125}I was expressed as d.p.m. mg^{-1} DNA and corrected for small variations in the injected dose by dividing by plasma ^{125}I - T_3 determined as that fraction of the plasma ^{125}I content precipitable by trichloroacetic acid²². The experiment with T_3 and the two experiments with SK&F L-94901 (dotted and solid lines) were done on separate occasions. The symbols represent the means of three hearts or two livers.

Table 1 A comparison of the potencies and receptor binding properties (*in vitro* and *in vivo*) of various substituted thyronines

SK&F No	R'	R''	Relative potency	Heart		Relative potency	Liver	
				Relative receptor binding <i>in vitro</i>	<i>in vivo</i>		Relative receptor binding <i>in vitro</i>	<i>in vivo</i>
T ₃	I	I	100	100	100	100	100	100
L-92904		I	0.5	13	0.3	2.2	15	5.1
L-93236		I	0.1	56	0.7	2.2	17	19
L-93993		I	Inactive	2.7	0.2	1.7	2.3	10
L-94690		I	0.1	4.0	0.4	2.4	2.0	38
L-94901		Br	0.1	2.0	1.3	18	0.9	50

Male, Wistar rats were rendered hypothyroid by administering 0.3 g l^{-1} of the antithyroid agent 2-mercapto-1-methylimidazole (MTI) in drinking water for at least 4 weeks before use. Single doses of thyronines were given intramuscularly in a vehicle of $0.15 \text{ M NaCl}/0.01 \text{ M NaOH}$ (1 ml kg^{-1}) 48 h before the GPGH activities of hearts and livers were determined. A standard '2+2' method was used to determine the potency of SK&F L-92904 and L-93236. Two doses of either T₃, SK&F L-92904 or L-93236 were administered, dose ratios were determined for hearts and livers, and relative potencies calculated. For SK&F L-93993, L-94690 and L-94901, as well as T₃, six doses were administered. For hepatic GPDH the resulting dose response curves were fitted to a hyperbolic curve by a non-linear curve fitting procedure and the mean effective dose (ED₅₀) calculated as described¹⁹. Potency is defined as the reciprocal of the ED₅₀; relative potency is the ratio of the potency of the novel compound to that of T₃, expressed as a percentage. The ED₅₀ for T₃ on cardiac GPDH activity was $0.7 \times 10^{-7} \text{ moles kg}^{-1}$; on hepatic GPDH it was $1.6 \times 10^{-7} \text{ moles kg}^{-1}$. All 6 compounds caused the same maximal increase in hepatic GPDH and are thus full agonists. Only SK&F L-92904 caused the same maximal increase in cardiac GPDH as did T₃. The highest dose used of the remaining compounds was 50 mg kg^{-1} ($77\text{--}90 \text{ } \mu\text{mol kg}^{-1}$). For SK&F L-93236, L-94690 and L-94901 only this dose significantly increased cardiac GPDH to about 50% of the level achieved with a maximally active dose of T₃. The potencies for these three compounds are, therefore, an approximation based on this figure and the assumption that the compounds would be full agonists if a high enough dose could have been administered. Even at a dose of 50 mg kg^{-1} SK&F L-93993 had no effect on cardiac GPDH. Receptor binding *in vitro* was determined using isolated nuclei. Briefly, nuclei, prepared by sedimentation of homogenates through high-density sucrose solutions, were incubated with ^{125}I -T₃ and increasing concentrations of non-radioactive T₃ or novel compound using published conditions for liver²⁰ and heart²¹. Bound and free ^{125}I -T₃ were separated by centrifugation and the concentration of non-radioactive ligand reducing the binding of ^{125}I -T₃ by 50% (IC₅₀) was determined. The affinity of T₃ for these receptors (determined from Scatchard plots) was about 10^9 M^{-1} for both heart and liver. *In vivo* receptor binding studies were conducted as described (Fig. 1, legend). Relative receptor binding is defined as the ratio of either the IC₅₀ or the ED₅₀ for T₃ to that for novel compound expressed as percentage.

and hepatic nuclei¹³ to examine the relationship between structure and affinity, and to optimise receptor binding. Indirect evaluations of the capacity of compounds to occupy receptors *in vivo* have also been made using their ability to compete with ^{125}I -T₃ for uptake by nuclei when both ^{125}I -T₃ and the compound are administered simultaneously to rats¹⁴.

Selective thyromimetics

We used the natural hormone, T₃, as the starting point and have confirmed that (1) a diphenylether core structure carrying a 4'-OH and 3,5-halogens is essential for high receptor affinity and (2) 3'-alkyl substituent is an effective replacement for a 3'-iodine atom in T₃ (ref. 15). We then tried to define the optimum 3'-substituent properties for high receptor affinity and hormonal activity. The 3'-benzyl analogue (SK&F L-92904) was made to test the effect of increasing substituent lipophilicity and/or size. This compound had 2.2% of the activity of T₃ in the liver but only 0.5% of the activity of T₃ in the heart. Its receptor affinity is greater, however, being 13% of that of T₃ for isolated cardiac nuclei and 15% for isolated hepatic nuclei (Table 1). This could be a consequence of limited solubility, so the potentially more soluble 4-hydroxybenzyl analogue, SK&F

L-93236, was made. This compound showed clear selectivity, being similar in activity to SK&F L-92904 as a hepatic thyromimetic (2.5% of T₃), but much weaker (0.1% of T₃) in the heart (Table 1).

The selectivity of these two compounds is not due to their receptor affinities. The affinities of SK&F L-92904 for the heart and the liver nuclear binding sites are essentially the same, and SK&F L-93236 actually has a higher affinity for cardiac receptors than for those from the liver. Both compounds were less active than would be predicted from their affinities for the receptors *in vitro*. To investigate these anomalies we conducted *in vivo* binding studies with SK&F L-92904 and SK&F L-93236. The results (Table 1) showed that both compounds were much less effective in preventing ^{125}I -T₃ binding to heart nuclei than to liver nuclei. If we assume that the ability of these T₃ analogues to depress nuclear binding of ^{125}I -T₃ *in vivo* is a measure of their capacity to bind to and activate the nuclear thyroid hormone receptor, then the reduced *in vivo* nuclear binding in the heart compared with the liver is broadly consistent with the observed selective thyromimetic activity. We have termed this differential effect on *in vivo* nuclear binding 'selective access'.

A larger group of related compounds was made; the pyridone derivative (SK&F L-93993) was found to be a full agonist in

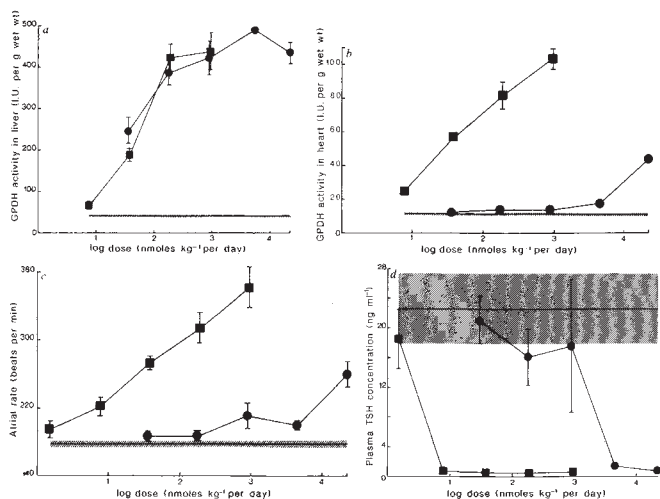


Fig. 2 Chronic treatment of hypothyroid rats with T₃ or SK&F L-94901. Effect on: *a*, hepatic GPDH activity; *b*, cardiac GPDH activity; *c*, spontaneous rate of beating of isolated right atria; *d*, plasma TSH concentration; ■, T₃; ●, SK&F L-94901.

Methods. Newly weaned, male, Wistar rats were rendered hypothyroid using MTI as in Fig. 1. They were then given seven daily consecutive intramuscular injections of either vehicle (0.15 M NaCl/0.01 M NaOH), or T₃ or SK&F L-94901 solutions in vehicle. The rats were anaesthetised with halothane 24 h after the final injection and blood samples withdrawn from the dorsal vena cava. Plasma was prepared and stored frozen at -20 °C until TSH could be measured by radio immunoassay (reagents were supplied by the NIH, Bethesda). The right atria were dissected out and mounted in Krebs-Henseleit saline buffered with CO₂/bicarbonate. Atrial rates were recorded after a 40-min stabilisation period at 33 °C. The remainder of the hearts and samples of liver were frozen on dry ice until their GPDH content could be determined as described¹⁹. Each point represents the mean of between 2 and 7 observations: the vertical bars are 2 s.e.m. Horizontal line, mean of values from rats treated with vehicle; cross-hatching, 2 s.e.m.

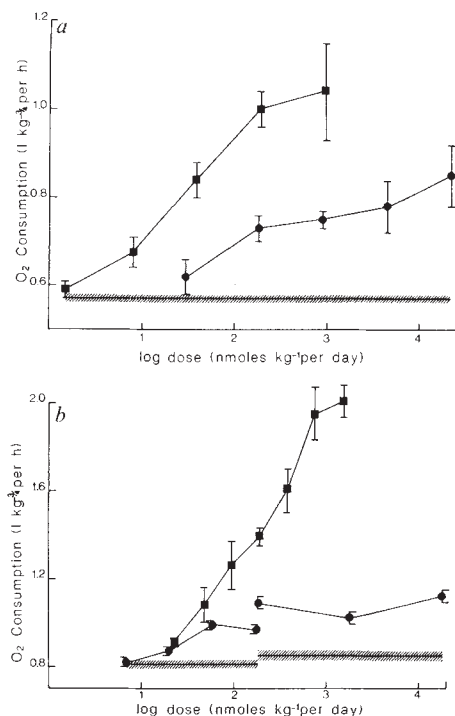


Fig. 3 The effects of T₃ and SK&F L-94901 on oxygen consumption. *a*, hypothyroid rats; *b*, normal rats; ■, T₃; ●, SK&F L-94901.

Methods. Rats were familiarised with the procedure for determining oxygen consumption to ensure that minimum values were obtained, then given seven daily consecutive doses of vehicle (0.15 M NaCl/0.01 M NaOH), T₃ or SK&F L-94901. Oxygen consumption was measured 24 h after the final dose and expressed as litres of O₂ (at Standard temperature and pressure (STP)) consumed kg^{-3/4} per h since this is independent of body weight²⁴. Symbols represent the means of between 3 and 7 rats; vertical bars, 2 s.e.m.; horizontal lines, mean oxygen consumption in vehicle-treated controls (n = 12 in *a* and 5 in *b*). *a*, hypothyroid rats, as in Fig. 2, given intramuscular injections at 1 ml kg⁻¹. Oxygen consumption, at 30 °C, was determined by a modification of the 'flow-through' technique²⁶ and was significantly greater than in controls for all groups except those given the lowest doses of either compound. *b*, normal rats dosed orally. Oxygen consumption was measured, at 29 °C, as described¹⁹. Three separate experiments over different dose ranges are shown on this graph. The mean oxygen consumption in the controls (n = 5) for the two experiments with SK&F L-94901 are shown as horizontal lines below the experiments with T₃ dose-response curve was intermediate at 0.84 ± 0.02 (4) l O₂ at STP kg^{-3/4} per h. Oxygen consumption was significantly greater than control in all treated groups except for those given the two lowest doses SK&F L-94901 and three lowest of T₃.

the liver but inactive in the heart (Table 1). Data from *in vivo* nuclear binding experiments also showed that all selective agonists exhibit selective access. Regression analyses suggest a dependence on 3'-substituent polarity and this prompted the synthesis of the more polar pyridazinone (SK&F L-94690), a selective thyromimetic showing a greater degree of selective access. But although selective access and selective activity is dependent on 3'-substituent hydrophilicity, the receptor affinity (and hence hormonal activity) was found to be correlated with high 3'-substituent lipophilicity. We therefore explored alternative structural modifications to increase activity. The 3,5-dibromo analogue (SK&F L-94901) was made because it was likely to be metabolically more stable than T₃ and was found to be highly selective and more potent than the diiodo analogue (Table 1). Like all other selective thyromimetics, SK&F L-94901 shows marked selective access (Table 1, Fig. 1).

Note that D-T₄ is not selective as assayed by induction of GPDH, having 7% and 11% of the activity of T₃ in heart and liver respectively.

The phenomenon of selective access implies that there is a different relationship between relative receptor binding *in vitro* and *in vivo* for the heart than for the liver. The significance of this is discussed below. But it is apparent from the data in Table 1 that there is no constant relationship between relative receptor binding *in vivo* and *in vitro*, nor between *in vivo* binding and overall potency for this group of compounds. The reasons for these differences have not been examined in detail, but many factors, as well as affinity for isolated receptors, control occupation and hence activation of receptors *in vivo*. These factors include binding to plasma proteins, metabolic transformations and the distribution of the thyronines between blood, organs

and cellular compartments. Finally the possibility that the compounds have differing efficacies cannot be ruled out.

The most active selective compound

After a single dose SK&F L-94901 has about 20% of the potency of T₃ in the liver and 0.1% in the heart (Table 1). Chronic treatment enhances the hepatic potency of SK&F L-94901. After seven daily intramuscular doses SK&F L-94901 is as potent as T₃ as a thyromimetic in the liver of hypothyroid rats, but still only had 0.1% of the activity of T₃ on cardiac GPDH activity and on the spontaneous rate of beating of isolated atria (Fig. 2*a*, *b* and *c*). In rats with normal thyroid function, given SK&F L-94901 or T₃ orally for seven days, a very similar result was obtained. The two compounds were equipotent in the liver but only T₃ had an effect on cardiac GPDH: SK&F L-94901 was inactive at a dose 200 times greater than the cardiac ED₅₀ for T₃. In this experiment the effects of SK&F L-94901 and T₃ on

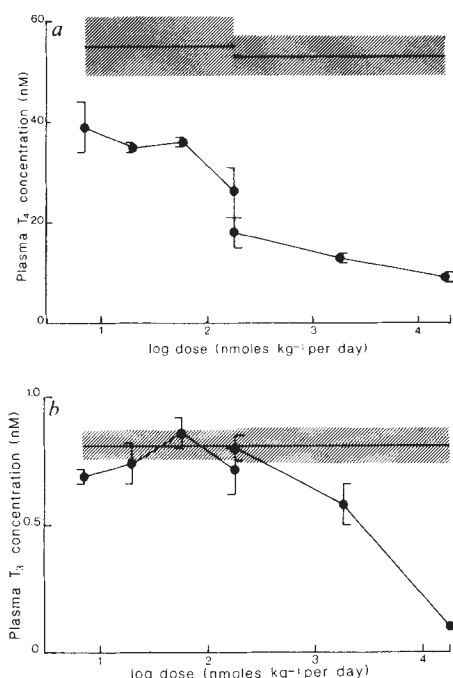


Fig. 4 Effect of SK&F L-94901 on *a*, plasma T₃ and *b*, T₄ concentrations. Male euthyroid rats were given seven daily consecutive oral doses of SK&F L-94901 and samples of blood from the dorsal aorta collected 24 h after the final dose under halothane anaesthesia. Plasma T₃ and T₄ concentrations were determined by radioimmunoassay (RIA) using commercially available kits (Amersham T₃ and T₄ RIA kits, Amersham) which are validated for use with rat plasma. Values shown are the means of 4 or 5 rats; vertical bars, 2 s.e.m.; horizontal lines, vehicle treated controls; cross-hatching, 2 s.e.m. of controls. Two separate experiments are shown; the control values for each experiment are shown above. *a*, T₃ concentrations. Only the highest dose of SK&F L-94901 had a statistically significant effect and this was to reduce T₃ concentrations to below the limit of detectability (0.15 nM). *b*, T₄ concentrations. Mean values in all treated groups, except those given the lowest dose of SK&F L-94901, were significantly smaller than in vehicle-treated controls.

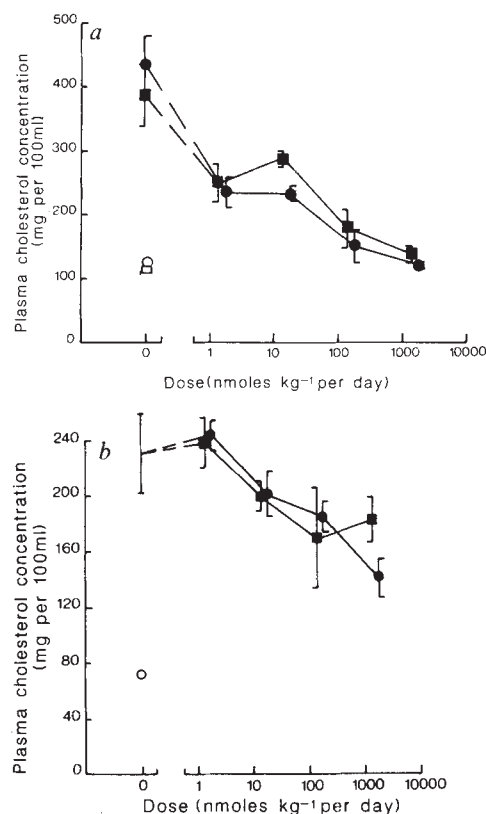


Fig. 5 Effects of T₃ and SK&F L-94901 on plasma cholesterol concentrations. *a*, Hypothyroid rats, fed with cholesterol. *b*, normal rats fed with cholesterol; ■, T₃; ●, SK&F L-94901; open symbols indicate groups fed with a normal diet. Male rats were rendered hypercholesterolaemic by feeding a diet containing 1.5% cholesterol and 0.5% cholic acid for 3 weeks. Seven daily consecutive oral doses (5 ml kg⁻¹) of vehicle (0.15 M NaCl/0.01 M NaOH), T₃ or SK&F L-94901 were given. Groups of male rats receiving a normal diet were also dosed with vehicle for seven days. Blood was taken under anaesthesia 24 h after the final dose and total plasma cholesterol measured with commercially available kit (Diagnostica Merck; E. Merck) using cholesterol oxidase. The symbols represent the mean values from groups of 4–5 rats; the vertical bars are the s.e.m.

renal GPDH were also measured; both compounds were fully active and roughly equipotent.

Chronic administration of either SK&F L-94901 or T₃ increases the minimal WBOC of rats, although the character and magnitude of the responses are very different. In hypothyroid rats the highest dose of T₃ used roughly double the WBOC. SK&F L-94901 increases the WBOC by 50% at its highest dose, with a shallower dose-response curve (Fig. 3*a*). This phenomenon was even more apparent in rats with normal thyroids, where the maximum response to SK&F L-94901 was about one quarter of that to T₃, and the dose-response curve to SK&F L-94901 was essentially flat over a considerable dose-range (Fig. 3*b*). This would be expected if a smaller number of organs gave a thermogenic response to SK&F L-94901 than to T₃ administration.

Both SK&F L-94901 and T₃ diminish plasma thyroid stimulating hormone (TSH) concentrations in hypothyroid rats after chronic treatment, but have dramatically different potencies. The lowest dose of SK&F L-94901 that reduced plasma TSH significantly was more than 1,000 times greater than the lowest effective dose of T₃ (Fig. 2*d*). When administered chronically to rats with normal thyroids only the highest dose of SK&F L-94901 reduces plasma T₃ concentrations significantly (Fig. 4*a*). This is similar to the lowest dose that significantly reduced plasma TSH in hypothyroid rats, implying that the effect of SK&F L-94901 on plasma T₃ could have been mediated by an action on pituitary thyrotrophs. By contrast, SK&F L-94901 reduces circulating T₄ significantly in a dose-related

manner, although no dose eliminated T₄ altogether (Fig. 4*b*). This maintenance of plasma T₃ while T₄ concentrations decrease is unusual and could be due to increased conversion of T₄ to T₃ or diminished elimination of T₃.

Unlike man, normal rats have very low circulating plasma cholesterol concentrations (about 50 mg per 100 ml), and almost all of this is in the high-density lipoprotein fraction (HDL). Thyroid hormones do not reduce total plasma cholesterol in this model. Hypocholesterolaemic activity was therefore determined in rats with artificially elevated plasma cholesterol produced by feeding a cholesterol-rich diet. Ultracentrifugation of plasma from cholesterol-fed rats revealed a discrete peak of cholesterol in the HDL fraction and a more diffuse fraction of lower density (reported to contain β VLDL and LDL¹⁶). When hypercholesterolaemic hypothyroid rats were given SK&F L-94901 orally for 7 days the concentration of cholesterol in their plasma was reduced in a dose-related fashion. This was mainly due to a reduction in the lower density fraction leading to an increase in the proportion of cholesterol carried on HDL. A daily dose as low as 1 μ g per kg per day caused a significant reduction compared to a vehicle-treated control group. Higher doses reduced plasma cholesterol concentrations to those found in animals given a normal diet. T₃ was also active under these conditions and the two compounds were of similar potency (Fig. 5*a*). Rats with normal thyroids are less sensitive to both

T₃ and SK&F L-94901, but even under these circumstances, the two compounds have similar hypocholesterolaemic potency (Fig. 5b).

Discussion

Unlike many hormone agonists and antagonists, the organ selectivity of SK&F L-94901 does not appear to result from differences in the receptors in the relevant tissues, as cardiac and hepatic nuclear receptor affinities are very similar for several hundred compounds (unpublished data), strongly suggesting that the two receptors are identical. The evidence that interaction with the nuclear T₃-binding protein is an obligatory step in the action of these hormones is now very strong, but factors controlling the access of the hormone to the nuclear proteins may exert a strong influence on activity. Indeed Oppenheimer and Schwartz¹⁷ have recently shown that both the plasma membrane and the nuclear membrane do have such a role. In particular, their experiments suggested that the free concentration of T₃ inside the nucleus is much greater than that in the cytoplasm, and they propose that some sort of concentrating mechanism or 'nuclear pump' must be in operation. This nuclear pump could be the source of selective access if the liver was more active *in vivo* in pumping SK&F L-94901 into the nucleus than was the heart. A corollary of this would be that the nuclear pump does not operate in our *in vitro* binding assay. Alternatively, hepatic cytosol may contain higher concentrations of SK&F L-94901 than does cardiac cytosol—either because it is actively accumulated by hepatocytes, or excluded by cardiac myocytes.

The similar potencies of SK&F L-94901 as an hepatic thyromimetic and as an hypocholesterolaemic agent support the idea that the actions of thyroid hormones on the liver are, at least in part, responsible for their ability to reduce plasma cholesterol concentrations. This is corroborated by the finding that in cats with normal thyroid function and a genetically hypercholesterolaemic strain of rabbit¹⁸ heterozygous for an LDL-receptor deficiency, SK&F L-94901 can also reduce circulating plasma cholesterol levels by about 30%. Again, LDL-cholesterol was preferentially reduced, resulting in an increased HDL:LDL cholesterol ratio. Humans with normally functioning thyroid are relatively sensitive to the hypocholesterolaemic effects of T₃, doses of a few microgrammes per kilogramme of body weight having a significant effect². These doses are similar to those reducing plasma cholesterol in the hypercholesterolaemic hypothyroid rat and suggest that SK&F L-94901 could reduce plasma cholesterol in man (and perhaps prevent atherogenesis) at doses far below those having a direct cardiac effect, and which would have minimal or no activity on basal metabolic rate and the thyroid-pituitary axis. The necessary toxicological evaluations are under way to allow the administration of SK&F L-94901 to man.

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The *Tetrahymena* ribozyme acts like an RNA restriction endonuclease

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A shortened form of the Tetrahymena self-splicing ribosomal RNA intervening sequence acts as an endoribonuclease, catalysing the cleavage of large RNA molecules by a mechanism involving guanosine transfer. The sequence specificity approaches that of the DNA restriction endonucleases. Site-specific mutagenesis of the enzyme active site alters the substrate sequence specificity in a predictable manner, so that endoribonucleases can be synthesized to cut at a variety of tetranucleotide sequences.

THE *Tetrahymena* ribosomal RNA intervening sequence (IVS) is a catalytic RNA molecule or ribozyme. It mediates RNA self-splicing, excising itself from the large ribosomal RNA precursor, and then converts itself to a circular form^{1,2}. In these reactions, the splice sites and circularization sites can be viewed as intramolecular substrates for an enzymatic activity of the IVS RNA³.

This view is supported by studies of the L-19 IVS RNA, a

linear form of the IVS that lacks the first 19 nucleotides. Because it has no circularization sites, the L-19 IVS RNA cannot undergo intramolecular reactions³, but it retains activity and can catalyse cleavage-ligation reactions on other RNA molecules⁴. When provided with oligo(cytidylic acid) as a substrate, the L-19 IVS RNA acts as an enzyme with nucleotidyltransferase (poly(C) polymerase) and phosphodiesterase (ribonuclease) activities⁴. With 3'-phosphorylated oligo(C) substrates, the same ribozyme

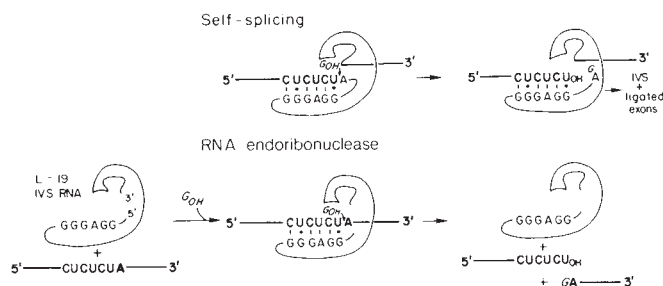


Fig. 1 A model for the endonuclease action of L-19 IVS RNA. The mechanism is an intermolecular version of the first step of pre-rRNA self-splicing. Thin letters and lines, IVS sequences; bold letters and thick lines, exon sequences (top) or substrate RNA sequences (bottom); the G in *italics* is a free guanosine nucleotide or nucleoside.

acts as a phosphotransferase and an acid phosphatase⁵. A key feature of all four of these reactions is the formation of a covalent enzyme-substrate intermediate in which a nucleotide or phosphate is esterified through the 3'-O of G414, the 3' terminal guanosine of the IVS RNA.

Here we describe a fifth enzymatic activity of the *Tetrahymena* ribozyme. It cleaves RNA molecules at sequences resembling the 5' splice site of the rRNA precursor. A free guanosine nucleotide is added to the 5' end of the downstream RNA fragment during cleavage. This allows one product to be end-labelled during the reaction. The reaction is analogous to the first step of pre-rRNA self-splicing (Fig. 1). Cleavage does not require nucleotide G414 of the ribozyme; thus unlike the first four activities, it does not involve formation of a covalent enzyme-substrate intermediate.

The ribozyme cleaves very specifically after the sequence CUCU; under stringent conditions it discriminates against sites that match three out of four of these nucleotides. This specificity approaches that of the DNA restriction endonucleases⁶. Site-specific mutations in the active site of the IVS RNA, the internal guide sequence^{7,8} or 5' exon-binding site⁹⁻¹¹, alter the sequence specificity of the ribozyme in a predictable manner. This will allow us to develop a set of sequence-specific RNA endonucleases that may be useful as tools for studying the molecular biology of RNA.

Sequence-specific cleavage of large RNAs

The L-19 IVS RNA enzyme was prepared by incubation of pre-rRNA under conditions that promote self-splicing, circularization, and site-specific hydrolysis^{3,4}. The 3'-terminal guanosine (G414) was then removed from the L-19 IVS RNA by periodate oxidation followed by β -elimination¹². As expected, the resulting ribozyme (L-19 IVS _{β}) has greatly reduced activity as a nucleotidyltransferase, assayed using [³²P]-p(C)₅ as a substrate (data not shown). But when GTP is added the ribozyme can cleave p(C)₅ and large RNA molecules. For example, the 504-nucleotide (nt) pAK105 transcript¹³, a fragment of mouse β -globin pre-mRNA containing the first intron, is cleaved to give major fragments of 148, 360 and 464 nt, and some minor fragments (Fig. 2a). As shown below, the 360- and 148-nt fragments can be explained as the 5' and 3' products of cleavage at position 360. The 464-nt fragment is the 3' product of cleavage at position 44, the 5' 44-nt fragment being too small to be observed. The fact that little 316-nt RNA (the expected product of cleavage at both position 44 and 360) is observed indicates partial digestion, with few molecules cleaved more than once.

Cleavage requires Mg²⁺ (optimum at 10–20 mM MgCl₂) and is essentially independent of monovalent cation in the range 0–200 mM NaCl. The pH optimum is between 7.5–8.0, and the temperature optimum is ~50 °C. Although β -eliminated L-19 IVS RNA catalyses the cleavage reaction, the enzyme works at the same rate whether or not G414 has been removed (data not

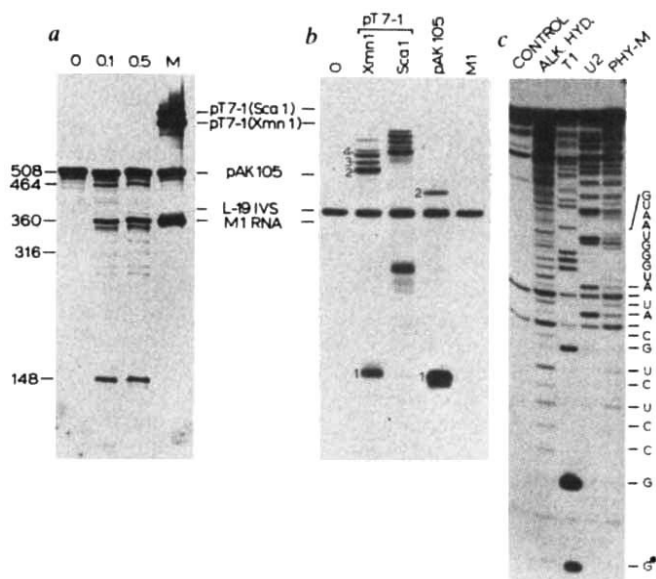


Fig. 2 The L-19 IVS _{β} RNA cleaves large RNA substrates with transfer of guanosine. *a*, Uniformly labelled 0.6 μ M pAK105 RNA (508 nt) incubated with 0.2 μ M L-19 IVS _{β} RNA and 0, 0.1 or 0.5 mM GTP (unlabelled) for 1 h under conditions described below. M, Mixture of 4 substrate RNAs as molecular weight markers. *b*, Various ³H-RNA substrates (1 μ g each) incubated with 0.2 μ M L-19 IVS _{β} RNA for 1 h, under the same reaction conditions as *a*, but with 120 μ M [α -³²P]GTP. The autoradiogram reveals [³²P]GTP-labelled products only. The L-19 IVS _{β} RNA also becomes ³²P-labelled during incubation. *c*, Nucleotide sequence of the cleavage product pAK105(1) determined by the enzymatic method³³. Some nucleotides could not be assigned due to the presence of a band in the untreated (control) RNA sample. G*, labelled GTP joined to the RNA during the reaction.

Methods. L-19 IVS RNA was made by methods similar to those described⁴, except that a different RNA polymerase-template system was used. Plasmid pT7-TT1A3 (which contains a T7 RNA polymerase promoter, a 42-bp 5' exon, the entire 413-bp IVS, and an 82-bp 3' exon) was cleaved with *Eco*RI and transcribed with bacteriophage T7 RNA polymerase³⁴. The transcripts were incubated further under self-splicing, circularization, and site-specific hydrolysis conditions to produce L-19 IVS RNA^{3,4}. The 3'-terminal guanosine was then removed by periodate oxidation and β -elimination¹² to yield L-19 IVS _{β} RNA. Substrate RNAs were produced by T7 RNA polymerase transcription of *Bam*HI-cleaved pAK105 (ref. 13), *Xmn*I- or *Sca*I-cleaved pT7-1 (purchased from U.S. Biochemical Corp.), and *Sna*BI-cleaved pDW27, which encodes M1 RNA (obtained from D. Wahl and N. Pace). Substrate RNAs were incubated with L-19 IVS _{β} RNA in 5 mM MgCl₂, 10 mM NaCl, 50 mM Tris-HCl, pH 7.5 at 50 °C; GTP was present at the concentration indicated. Reactions were stopped by adding EDTA to a final concentration of 25 mM. Products were analysed by electrophoresis in 4% polyacrylamide, 8 M urea gels and subjected to fluorography (*a*) or autoradiography (*b*).

shown). We explain this by supposing that, at saturating GTP concentrations, the attack by GTP on the substrate competes very effectively with attack by G414.

When [α -³²P]GTP is included in the reaction of pAK105 RNA, the 148- and 464-nt cleavage products are labelled (Fig. 2b). Direct sequencing of these labelled RNA fragments (Fig. 2c) showed that cleavage and GTP-addition occur at nucleotides 44 and 360 in the sequence, such that the downstream cleavage products are 5'-GTP labelled. The bonds formed by GTP addition are sensitive to RNase T₁, confirming that the GTP is covalently added through its 3'-O by a normal 3'-5' phosphodiester bond. Reaction of the 841-nt pT7-1 (*Xmn*I) RNA (essentially pBR322 sequences) produced four major labelled fragments. These were sequenced in the same manner. The pT7-1 RNA with an extra 122 nt at its 3' end was produced by transcription of pT7-1 DNA that had been cleaved at the *Sca*I site; the

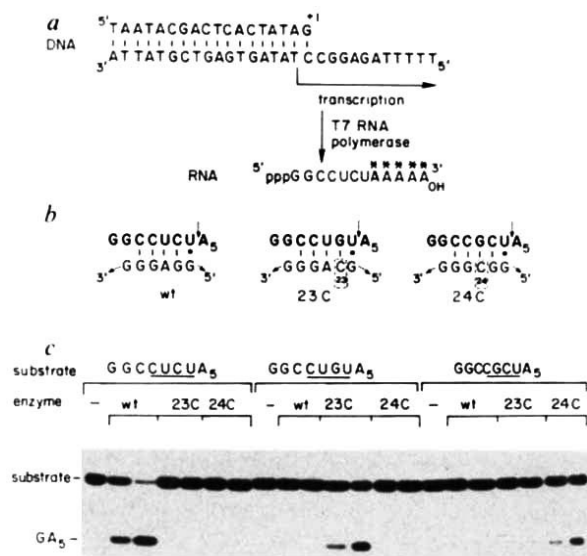


Fig. 3 Three different ribozymes can distinguish between sequences that differ by a single base change in the recognition element. *a*, Synthesis of defined oligoribonucleotide substrates by the method of Lowary *et al.*¹⁹. DNA was transcribed with T7 RNA polymerase in the presence of [α -³²P]ATP to produce oligoribonucleotides labelled with ³²P in the positions indicated (*). *b*, Proposed interactions between the three oligoribonucleotide substrates (top strands, bold) and the active sites of the matched ribozymes (bottom strands). Arrows, sites of cleavage and guanosine addition. *c*, Cleavage of three oligoribonucleotide substrates by wild-type and variant L-19 IVS β RNAs, assayed by 20% polyacrylamide, 7 M urea gel electrophoresis. (—), untreated substrate. Lanes are run in pairs; 1.0 μ M ³²P-labelled substrate was incubated with 0.125 μ M ribozyme for 15 min (left lane) or 60 min (right lane) at 50 °C in 10 mM MgCl₂, 10 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM GTP (unlabelled), 2.5 M urea. Because the ³²P is confined to the nucleotides downstream from the cleavage site, only the smaller product is apparent. The identity of the GA₅ product was confirmed by treatment of the substrate and reaction mixtures with RNase T₂ and monitoring the transfer of ³²P to Gp. A band migrating above GA₅ in lanes 2 and 3 appeared in some experiments but has not been identified.

Methods. Substrates were prepared by transcription of deoxy-oligonucleotides synthesized on an Applied Biosystems DNA Synthesizer. The same promoter top strand was used in each transcription. The bottom strand, which contains promoter and template sequences, was varied to obtain the different RNA substrates. The DNA was transcribed with purified phage T7 RNA polymerase³⁴ as described¹⁹. Variant ribozymes are described by Been and Cech¹¹. The L-19/23C ribozyme was prepared by transcription of pBG/-2G:23C and treatment of RNA under site-specific hydrolysis conditions¹¹. The 24C ribozyme was similarly prepared from transcripts of pBG/-3G:24C. The variant ribozymes were not subjected to β -elimination to remove their 3'-terminal G.

Table 1 Sites of cleavage of large RNA substrates by the wild-type L-19 IVS β RNA

Substrate	Site	Size (nt)*	Sequence†
pAK-105 (β -globin pre-mRNA)	1	148	UCCUCU ⁴ GCCUC
	2	464	AACUCU AAGAG
pT7-1 (pBR322)	1	145	UCCCUU UUUUG
	2	556	CACUCU CAGUA
	3	603	UAUUUU CUCCU
	4	805	UCCUCU AGAGU
Consensus observed			U ^C CUCU ⁴ N
expected‡			C ^C CUCU ⁴ N

* Size of the GTP-labelled fragment.

† Sequences are listed in 5' $\rightarrow$ 3' direction. Arrows indicate sites of cleavage and guanosine addition.

‡ The expected consensus sequence is based on the known sequence at the end of the 5' exon (CUCUCU) modified by the possibility that either a C or a U at position -5 might be able to pair with the G in the active site. At position -1, C may work less well than U^{7,27,35}.

pAK105 RNA, only one CUCU site was not cleaved. (Cleavage at this site would produce a labelled 378-nt RNA.) Many sites that match CUCU in three out of four positions were not cleaved. These include 17 CUMU sequences (where M $\neq$ C) and 7 CNCU sequences (where N $\neq$ U). In pT7-1 RNA, cleavage was observed at both of the CUCU sequences in the RNA, but at only one of the 15 UUUU sequences present. Thus, the ribozyme has a strong preference for cleavage after the tetranucleotide CUCU.

M1 RNA, the RNA subunit of *Escherichia coli* RNase P¹⁶, was not cleaved by L-19 IVS β RNA under standard reaction conditions (Fig. 2*b*). M1 RNA contains the sequence UCCUCU, which should be an excellent target site. However, this sequence is part of a stable hairpin stem^{17,18}, which presumably makes it unavailable as a substrate. Denaturation of M1 RNA with glyoxal allows this site to be cleaved by L-19 IVS β RNA (unpublished data), in support of the interpretation that RNA secondary structure can inhibit cleavage.

Active-site mutations alter substrate specificity

We then studied the substrate specificity in a defined system where the substrate RNA had no secondary structure that might affect the cleavage reaction. Oligoribonucleotide substrates were made using the phage T7 RNA polymerase transcription method (Fig. 3*a*). One substrate contained a perfect match to the tetranucleotide consensus sequence. Two other substrates had single-base changes giving a three-out-of-four match to the consensus.

These substrates were tested with the wild-type L-19 IVS β RNA and with two altered ribozymes. The two variants have single-base changes in the 5' exon-binding site that alter the sequence specificity of the first step in pre-rRNA self-splicing¹¹. The 23C variant (G $\rightarrow$ C at position 23 of the L IVS RNA) is expected to recognize CUGU substrates, and the 24C (A $\rightarrow$ C at position 24) variant should recognize CGCU (Fig. 3*b*). In the presence of 2.5 M urea, each substrate is cleaved exclusively by the ribozyme that can make a perfectly base-paired enzyme-substrate complex (Fig. 3*b, c*). We find that 2.5 M urea or 15% formamide greatly increases specificity, allowing each ribozyme to differentiate between substrates with a single base change in the recognition sequence. Our current model is that these denaturants destabilize the base-pairing between the substrate and the active site nucleotides of the ribozyme, thereby discriminating against mismatched complexes.

When variant ribozymes were incubated with the 504-nt pAK105 RNA, each ribozyme gave a different pattern of cleavage products (data not shown). One major cleavage site was mapped for three variant ribozymes, including a 25C variant (G $\rightarrow$ C at position 25). The sites cleaved by the 23C, 24C and

labelled products increased in molecular weight as expected. In all reactions, including those in which substrate RNA was omitted, the L-19 IVS β RNA became labelled, perhaps by guanosine exchange^{14,15}. The sites of self-labelling were heterogeneous.

The sequence near the 5' end of each end-labelled product was compared with the known sequence of the RNA to identify the nucleotides preceding the site of cleavage (Table 1). All the cleavage sites are preceded by four pyrimidines; the consensus sequence is CUCU. This is exactly the tetranucleotide sequence expected to be an optimal target for the ribozyme. The importance of nucleotides at -5 and -6 from the cleavage site is not clear, but the absence of G residues may be significant. There is no apparent sequence preference downstream from the cleavage site; all four nucleotides are found at position +1.

The potential sites in the RNA that were not cleaved by the ribozyme also provide information about the specificity. For the

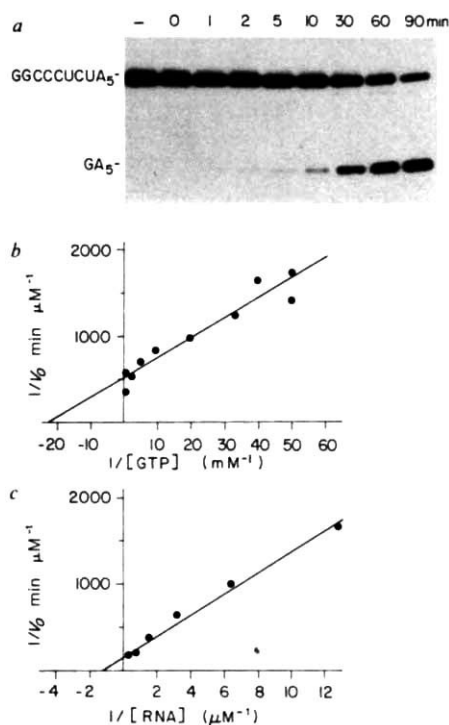


Fig. 4 Kinetic analysis of the RNA endoribonuclease reaction. *a*, The oligoribonucleotide substrate ($2.5 \mu\text{M}$) was incubated with wild-type L-19 IVS $_{\beta}$ RNA ($0.2 \mu\text{M}$) as in Fig. 3, except that urea was omitted. *b*, Kinetics of cleavage of GGCCUCUA $_5$ as a function of GTP concentration. RNA substrate concentration was kept constant at $2.5 \mu\text{M}$. *c*, Kinetics of cleavage as a function of RNA substrate concentration, with GTP concentration kept constant at 0.5 mM .

Methods. Products were separated by polyacrylamide gel electrophoresis. Each gel was cut into strips using the autoradiogram as a guide and the radioactivity in the unreacted substrate and the GA $_5$ product was determined by liquid scintillation counting. The initial rate of cleavage (v_0) was determined from a semilogarithmic plot of the fraction of reaction as a function of time. The graphs are linear least-squares fits to a plot of $1/v_0$ against inverse substrate concentration.

25C ribozymes are preceded by CCCUGU, UCUGCU, and CUGUCU, respectively; the underlining indicates the base that forms a G·C base pair with the mutated nucleotide in the variant ribozyme. Each of these sequences can form 6 continuous base-pairs with the active site nucleotides of the variant ribozyme.

Cleavage is catalytic

The time course of cleavage of the oligonucleotide substrate GGCCUCU↓AAAAA (the arrow designates the cleavage site) by wild-type ribozyme is shown in Fig. 4*a*. The reaction of $2.5 \mu\text{M}$ substrate with $0.2 \mu\text{M}$ ribozyme is 66% complete in 90 minutes. Thus, it is readily apparent that the ribozyme is acting catalytically.

The reaction rate was determined at a series of substrate concentrations. The kinetics are adequately described by the Michaelis-Menten rate law. The dependence of the rate on GTP concentration is shown in the form of a Lineweaver-Burk plot in Fig. 4*b*. The K_m for GTP is $44 \mu\text{M}$. The dependence of the rate on RNA substrate concentration at saturating GTP concentration is shown in Fig. 4*c*. The K_m for this oligoribonucleotide substrate is $0.8 \mu\text{M}$, and k_{cat} is 0.13 min^{-1} . Thus under V_{max} conditions the enzyme turns over about 8 times per hour.

Discussion

We find that the L-19 IVS RNA acts like an RNA restriction endonuclease. It catalyses the sequence-specific cleavage of other RNA molecules while transferring guanosine to the downstream RNA fragment. The enzyme has a strong preference for cleaving RNA after the sequence CUCU (Table 1), and in a solution containing 2.5 M urea it cleaves after CUCU, but ignores the related sequences CUGU and CGCU (Fig. 3*c*). Additional sequences must be tested to provide a complete picture of the sequence specificity of the ribozyme, and to examine the effect of the nucleotides at positions -5 and -6 on the efficiency of cleavage. Its specificity may approach that of the DNA restriction endonucleases, most of which recognize a particular 4 or 6 base-pair sequence.

The endoribonuclease reaction is analogous to the first step of pre-rRNA self-splicing (Fig. 1). Both the enzymatic and the self-splicing reactions use the same two binding sites, an oligopyrimidine-binding site and a guanosine-binding site, to achieve specificity and to contribute to catalysis. The oligopyrimidine-binding site is also known as the 5' guide sequence⁸ or the 5' exon-binding site⁹⁻¹¹. Its role in self-splicing has been defined by analysis of single-base mutations and second-site suppressor mutations^{8,11,20}. The same nucleotides bind the substrate in the endoribonuclease reaction, as shown by the change in substrate specificity of the mutant enzymes (Fig. 3). The altered specificity follows the rules of Watson-Crick base pairing. The general features of the guanosine-binding site involved in self-splicing have been described^{21,22}, but the site has not been localized to a particular set of nucleotides. The endoribonuclease activity appears to use the same guanosine-binding site, as in both cases guanosine is as active as GTP, whereas UTP, CTP, ATP and dGTP have little if any activity (data not shown). In addition, the K_m of $44 \mu\text{M}$ for GTP observed in this study agrees reasonably well with the value of $32 \pm 8 \mu\text{M}$ determined for self-splicing under somewhat different reaction conditions²².

The endoribonuclease activity of L-19 IVS RNA does not require the 3'-terminal guanosine (G414). In this respect it differs from the nucleotidyl transfer, phospho transfer and hydrolytic activities, in which a covalent enzyme-substrate complex, apparently necessary for reaction, is formed through G414^{4,5}. Thus the L-19 IVS RNA is not restricted to reaction mechanisms involving formation of a covalent enzyme-substrate intermediate. It can also catalyse bisubstrate reactions by a single-displacement mechanism. Ribonuclease P, an RNA enzyme that catalyses cleavage by hydrolysis rather than by transesterification, also appears to act without formation of a covalent intermediate^{23,24}.

The L-19 IVS RNA endoribonuclease activity reported here appears to require single-stranded RNA substrates. Work recently reported by Szostak²⁵ suggests that a smaller version of the *Tetrahymena* IVS RNA, lacking the 5' exon-binding site, may have an endoribonuclease activity that requires a base-paired substrate. The substrate tested²⁵ was an RNA fragment containing the end of the 5' exon paired with the 5' exon-binding site. However, this RNA 'substrate' included a substantial portion of the IVS RNA, so it is unclear whether the ribozyme has endoribonuclease activity with double-stranded RNA substrates in general.

The enzymatic cleavage reaction has some implications for *Tetrahymena* pre-rRNA self-splicing. The observed sequence-specificity of the enzymatic reaction provides further evidence that the CUCU sequence immediately preceding the 5' splice site is both necessary and sufficient to designate the site. RNA structure models^{7,26,27} and data from the *trans*-splicing reaction^{9,10} and genetic studies^{8,11,15} also support this conclusion. Now that the first step of self-splicing can be mimicked using a short oligonucleotide substrate, analysis of this step should become easier. For example, the uridine nucleotide immediately preceding the site of guanosine addition can be replaced with

modified uridine. This uridine is conserved in Group I intervening sequences^{7,27}; modifications would allow detailed investigation of the interactions involving this uridine.

Sequence-specific endoribonucleases may be useful in the study of RNA, in ways analogous to DNA restriction endonucleases⁶. The pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be cleaved to fragments of a size more useful for study. The 4-nucleotide specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence; an RNA of random sequence would have an average of 1 cleavage site every 256 bases. The automatic end-labelling of one fragment during ribozyme cleavage is a practical advantage.

Ribozyme development has only just begun. The cleavage efficiency needs improving, so that digestion is complete. Denaturants such as urea and formamide appear to increase the specificity of cleavage; they should also melt the secondary structure of the substrate and maximize the availability of target sequences. There are 256 possible tetranucleotide cleavage enzymes of which we have investigated only three. Further mutagenesis experiments are needed to discover how many of these are both efficient and specific.

Protein ribonucleases cleave RNA substrates with high specificity by recognizing a combination of RNA structure and sequence; the structure is more important (for instance RNase III (ref. 28) and RNase M5 (ref. 29)). But proteins that cleave single-stranded RNA substrates are only specific for mononu-

cleotides or dinucleotides (for instance, RNase T₁ cleaves after guanosines³⁰). Thus, the L-19 IVS RNA is considerably more sequence-specific than any known protein ribonuclease.

RNA sequence recognition usually involves recognition of RNA by RNA. Triplet codons in messenger RNA are recognized by anticodons in transfer RNA, and ribosome binding sites in prokaryotic mRNA are recognized using the Shine-Dalgarno interaction with 16S rRNA. It is easy to imagine uses for sequence-specific protein ribonucleases in pre-mRNA splicing and 3'-end generation, but there is no evidence for such enzymes. Instead, 5' splice sites in pre-mRNA are recognized using the U1 small nuclear RNA^{13,31} and the 3'-end cleavage site of histone H3 pre-mRNA by U7 snRNA³²; much of the sequence discrimination is provided by complementary base-pairing. Perhaps it is difficult to construct an active site in a polypeptide to bind an unstructured stretch of nucleotides specifically. It is possible that in certain reactions involving RNA substrates, RNA catalysis has not been superseded by protein catalysis for the simple reason that RNA does the job better.

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LETTERS TO NATURE

Is CO₂ responsible for the outbursts of comet Halley?

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Outbursts are thought to originate from the nucleus of comet Halley; cameras on the Vega and Giotto spacecraft have recorded the images of jets^{1,2}, and rapid fluctuations are seen in the visible light curve, detected, for example, by the International Ultraviolet Explorer satellite during the time the spacecraft were making their observations³. This activity does not appear to be limited to when the comet is near perihelion, but has been reported for heliocentric distances >4.6 AU pre-perihelion⁴. The outbursts are probably triggered by the heating of relatively volatile material within the

ice. We report here spectroscopic data from the IUE showing a strong correlation between a visible outburst in the near-nucleus region on 18.8 March 1986 and the sudden appearance of a large column abundance of CO₂⁺ ions in the tail. These data suggest that CO₂ may play a significant role in the outburst process. This outburst may have been the source of the detached clumps of CO⁺ emission recorded by ground-based observers on 19 and 20 March 1986^{5,6}.

Data were obtained during a combined 16 h US1-US2 IUE shift on 18 March 1986 beginning at 11:00 UT. Observations consisted of a large number of relatively short exposures taken at various offset distances from the nucleus with the long-wavelength (LWP) spectrograph to map the spatial distribution of the OH emission farther from the comet than previously⁷ and thereby determine the OH scalelength. An initial set of exposures was taken with all the offsets (the maximum was 8 arc min) on the sunward side of the nucleus along the Sun-comet line. To examine the symmetry of the OH coma, subsequent exposures consisted of sunward-tailward pairs at equal distances, again along the Sun-comet line. Three short exposures at the nucleus were also obtained; at the beginning, middle and end of the observational period. Near the middle of the period, a moderately long exposure of the LWP spectrograph was carried out

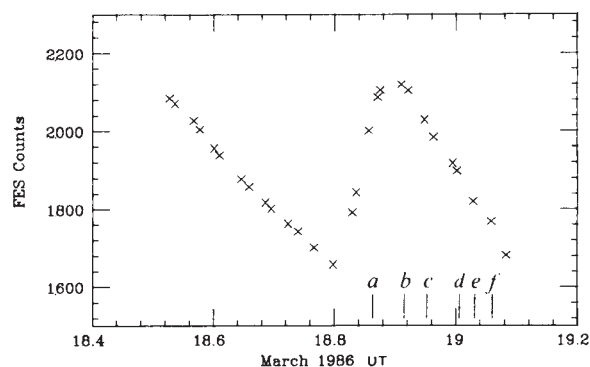


Fig. 1 Light curve of comet Halley for 18, 19 March 1986 derived from the IUE Fine Error Sensor. The effective aperture is ~ 18 arc square. *a-f*, Times of the spectrograph exposures listed in Table 1.

to determine the flux of the continuum and weaker features such as CS and C_2 .

The IUE fine error sensor (FES) is a slit-jaw camera used to acquire and track a desired target. The FES can also be used as a visible light photometer (wavelength range $\sim 4,000$ – $6,500$ Å) sensitive to dust and C_2 and CN emission in a region ~ 18 arc square ($11,000$ km $\times$ $11,000$ km) around the centre of brightness of the comet. It can provide continuous monitoring of short-term variability⁸ not possible at ground-based observatories. The FES data from March 18, 19 are shown in Fig. 1, and indicate an outburst beginning at $\sim 19:22$ UT, increasing linearly and reaching a maximum about 2.5 h later, and then decaying at a faster rate than before the outburst. The mid-point exposure times of the spectra presented in Table 1 are also shown (*a-f*). At the time of the outburst, the heliocentric distance of Halley was 0.98 AU, the geocentric distance was 0.84 AU; the solar elongation was 63.7° and the phase angle was 66° . Foreshortening along the Sun-comet line was $<10\%$.

Spectra *a-c* are shown in Figs 2 and 3. Spectrum *b* clearly shows the $\tilde{B}-\tilde{X}$ doublet of CO_2^+ at $2,884$ – $2,896$ Å, at a flux comparable to the nearby OH (1,0) band at a position 4 arc min, or $150,000$ km projected on the sky, on the tailward side of the nucleus. The CO_2^+ feature is absent at the same distance on the sunward side (*c*). It is still present (despite the higher camera noise level from the shorter exposure) 1 arc min tailward in *d*, taken 2.25 h after *b*. In the spectrum at the nucleus (*a*) the CO_2^+ emission is masked by the very strong continuum of dust-scattered sunlight, and accounts for only $\sim 15\%$ of the observed emission at $2,890$ Å. Festou *et al.*³ have suggested, from earlier IUE spectra of Halley, that this comet appears to show a much lower emission rate of CO_2^+ relative to OH than several other comets observed by the IUE⁹. However, as Fig. 2*a* shows, the region of the CO_2^+ doublet contains a maximum in the spectrum of scattered solar radiation (dotted line) which masks the CO_2^+ feature. Subtraction of the solar spectrum, properly reddened¹⁰, clearly shows the CO_2^+ doublet (*b*).

The coincidence of the tailward exposures with the outburst was extremely fortuitous, as was another observation that showed the transient nature of the outburst. The spectra denoted by *d* and *e* in Table 1 were both taken at the same offset of 1 arc min into the tail because of an operational error. A comparison shows more than a fourfold decrease in a period of 37 min for the CO_2^+ emission, whereas the OH brightness remained nearly constant. Note also the decrease in CO_2^+ brightness between spectra *b* and *d* which is all the more striking as *d* was obtained with a fourfold smaller offset into the tail.

Although the spatial and temporal coverage afforded by the IUE spectrograph is extremely limited, the data strongly imply a sudden enhancement followed by a rapid decrease in the abundance of CO_2^+ ions in the plasma tail in the $\sim 10^4$ s after the onset of the outburst detected by the FES. The presence of CO_2^+ suggests the production of a large enhancement of CO_2

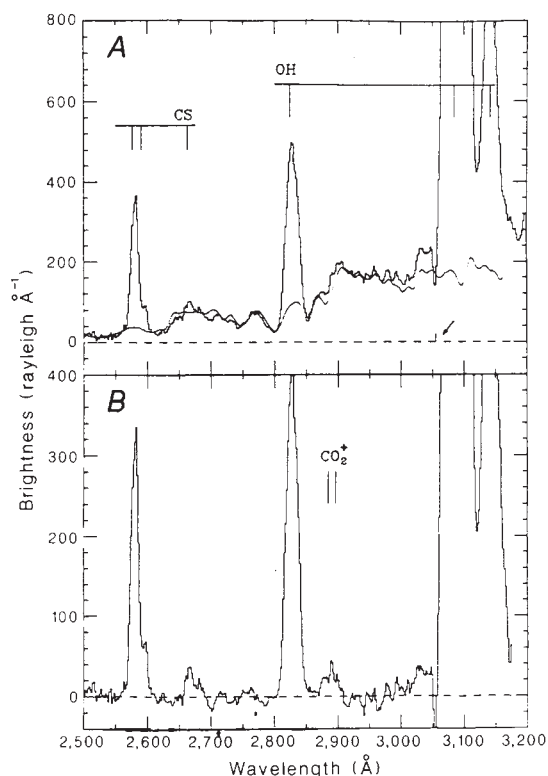


Fig. 2 *A*, spectrum *a*, at the centre of brightness, showing the prominent OH and CS emission features. The dotted line is a solar spectrum smoothed to the resolution of the IUE spectrograph. The position of a camera reseau mark is indicated by the arrow; *B*, same spectrum but with a reddened solar spectrum subtracted.

molecules at the nucleus, perhaps enough to drive the outburst in terms of propelling grains into the coma. A qualitative estimate of the CO_2 production rate required can be derived from the observed surface brightness of the CO_2^+ emission.

The IUE spectrograph slit is ~ 10 arc s $\times$ 20 arc s with the long axis $\sim 73^\circ$ to the Sun-comet line for these observations. This long axis is then part of a surface $150,000$ km from the nucleus through which all the ions produced inside must flow under the accelerating influence of the solar wind. The 20 arc s dimension corresponds to a linear width $w = 12,000$ km, and the uniform spatial distribution of the CO_2^+ emission in the aperture indicates that the width of the ion tail is considerably greater than this slit width. The average column density in the aperture, N , is given in terms of the measured surface brightness, B , in rayleighs, as:

$$N = \frac{Br^2}{g} \times 10^6 \text{ cm}^{-2}$$

where g is the fluorescence scattering efficiency in photons per second per molecule at 1 AU and r is the heliocentric distance of the comet in AU.

The rate of flow of CO_2^+ ions through that portion of the surface defined by the slit is

$$q = Nvw \text{ ions s}^{-1}$$

where v is the outflow velocity at the surface. To estimate a lower limit to v , if the ions observed in spectrum *b* were produced at the nucleus at the onset of the outburst, $9,300$ s earlier, their mean velocity into the tail would be 16 km s^{-1} . The assumption of uniform acceleration then gives $v > 32 \text{ km s}^{-1}$. Thus, in the IUE aperture, using $g(CO_2^+) = 2 \times 10^{-3} \text{ photons s}^{-1} \text{ ion}^{-1}$ (at 1 AU) (ref. 11 and J. L. Fox, personal communication), the observed brightness of 560 R gives $q > 1.0 \times 10^{27} \text{ s}^{-1}$. However, the width of the ion tail region is significantly greater than the $12,000$ km defined by the IUE aperture, so q must be even larger.

Table 1 IUE data from 18–19 March 1986

Sequence	Image no.	Exposure duration(s)	Mid-point time of exposure (UT)	Position	$B(\text{CO}_2^+)$ (R)	$B(\text{OH } 1,0)$ (R)	$B(\text{OH } 0,0)$ (kR)
—	LWP7823*	30	19:56	nucleus	—	7,000	385
a	LWP7824	900	20:43	nucleus	$750 \pm 260^\dagger$	saturated	saturated
b	LWP7825	360	21:57	4 arc min tailward	$560 \pm 20^\ddagger$	645	26.2
c	LWP7826	360	22:51	4 arc min sunward	-10 ± 25	760	31.7
d	LWP7827	90	00:08	1 arc min tailward	295 ± 95	2,340	106
e	LWP7828	90	00:45	1 arc min tailward	60 ± 75	2,370	105
f	LWP7829	90	01:27	1 arc min sunward	-115 ± 80	2,060	114
—	LWP7830	30	02:03	nucleus	—	7,300	359

* This spectrum is included because the OH bands are saturated in LWP7824.

† With continuum subtracted.

‡ Uncertainty is standard deviation in camera noise only.

Ground-based monochromatic images of other comets at similar heliocentric and geocentric distances¹², suggest that $w > 50,000$ km, so that the ion flow rate, which is equal to the CO_2^+ production rate, must be $> 4.2 \times 10^{27} \text{ s}^{-1}$.

For comparison, the water production rate, derived from the observed OH (0,0) band brightness³, is $3.9 \times 10^{29} \text{ s}^{-1}$. An upper limit to the CO_2 volume mixing ratio relative to H_2O , derived from the Giotto neutral mass spectrometer data¹³, is 0.035, and if this represents a 'quiescent' value, it would imply a production rate, $Q(\text{CO}_2) < 1.4 \times 10^{28} \text{ s}^{-1}$. The observed CO_2^+ emission rate would then require efficient ionization and dissociation of all neutral CO_2 produced within a region $< 10^4$ km from the nucleus. As the time scales for CO_2 destruction are significantly longer than 10^4 s, the only way to account for the required CO_2^+ production rate is by a large increase in $Q(\text{CO}_2)$ during the outburst over the quiescent value. Surprisingly, the OH (0,0) band emission rate does not show an increase after the outburst (see the first and last lines of Table 1), but, in fact, shows a slight decrease, despite the lifetime of the OH parent, H_2O (~ 1 day). Almost all the IUE data, except for this outburst, show a strong correlation between the FES count rate and the measured OH brightness¹⁴.

The ionization source near the nucleus remains an open question. Photoionization of CO_2 by solar extreme ultraviolet photons is relatively slow (the rate at 1 AU is $\approx 7 \times 10^{-7} \text{ s}^{-1}$)¹⁵ so that 10^4 s after the beginning of the outburst $< 10^{-2}$ of the CO_2 molecules produced would have been ionized. Electron impact ionization is also a possible source, particularly within $\sim 10^4$ km of the nucleus where significant concentrations of $10\text{--}10^3$ eV electrons were measured by the plasma experiment on Vega 1¹⁶. Evidence for electron impact excitation of forbidden ultraviolet transitions in the inner coma was also obtained in other ultraviolet observations of comet Halley¹⁷. However, although collisional ionization, in addition to photoionization, could significantly increase the CO_2^+ formation rate, at the required rate the entire neutral molecular coma (principally H_2O) would quickly be converted to ions and atoms contrary to all known observations.

Several ground-based observers^{5,6} have reported monochromatic images in CO^+ ($\lambda = 4,260 \text{ \AA}$) taken on 20 March 1986 showing a detached condensation of CO^+ at $\sim 2.6 \times 10^6$ km from the head of comet Halley. Pederson and Vio⁵ also determined the velocity of the condensation relative to fixed stars; the velocity relative to the comet nucleus was found to be $\sim 35 \text{ km s}^{-1}$. Extrapolating backwards from their observation time of UT 20.295 March 1986, and allowing for changes in velocity of the ion cloud, it is very likely that the origin of this cloud is the outburst detected by the IUE. Unfortunately, although CO^+ is detectable in the wavelength region of the IUE exposures listed in Table 1 through fluorescence in the First Negative system near $2,200 \text{ \AA}$ (ref. 18), the combination of low fluorescence efficiencies¹⁹ and low sensitivity of the IUE camera at $2,200 \text{ \AA}$ resulted in no detection of CO^+ emission in any of

the IUE spectra. However, as CO appears to be the second most abundant parent molecule in the coma of comet Halley¹⁷ and is even more volatile than CO_2 , it is likely that both species are released simultaneously during the outbursts (together with the grains that give rise to the increase in FES counts seen in Fig. 1) and both the IUE and ground-based observations can be associated with a common event. The presence of CO and CO_2 is also expected on the basis of laboratory experiments that have shown that both CO and CO_2 are likely to be present in comparable abundance in pre-solar cometary grains²⁰.

The role, if any, of the solar wind in the observed outburst is not clear. Measurements of solar wind parameters by the Sakigake spacecraft²¹, not too far from Halley, showed a large increase in electron density early on 18 March 1986. However, even at the higher particle flux, the rate for charge exchange ionization of molecules by solar wind protons is at best comparable to the photoionization rate, and the solar wind is stood off by mass loading from the inner coma region where most of the ionization must occur²². There is also no evidence in any of the sunward offset exposures taken by IUE before the outburst of

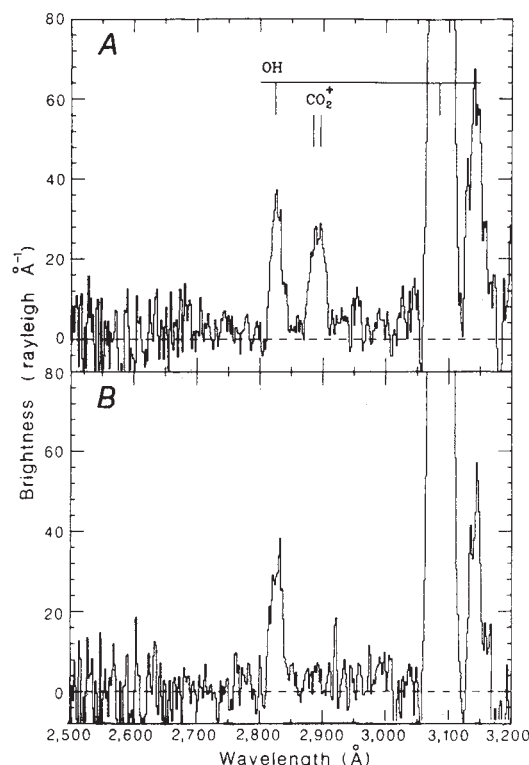


Fig. 3 Spectra b and c (from Table 1) (A and B respectively) taken 4 arc min tailward and sunward, respectively.

any CO_2^+ emission that would indicate enhanced ionization in the coma. Thus, while the solar wind may have significantly affected the structure of the ion tail once formed, it is unlikely to have initiated the outburst.

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Rocket ultraviolet spectroscopy of comet Halley and abundance of carbon monoxide and carbon

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The ultraviolet spectrum of a comet's coma is dominated by emissions from the dissociation products of water, OH, H and O, and from secondary species: C, C^+ , CO, CO^+ , CO_2^+ , S, and CS (ref. 1). We report here two far ultraviolet observations of comet Halley made on 26 February 1986 and 13 March 1986 with a sounding rocket experiment. This is the first time that long-slit spectroscopy, a standard technique for the study of extended objects such as comets and nebulae, has been applied to the far ultraviolet study of a comet. The observed CO spatial profiles can be modelled by a radial outflow model² for a parent molecule and suggest that the CO is produced directly from the nucleus of the comet. Using the observed O I emission profile to deduce the H_2O production rate, the abundance of CO relative to H_2O is found to be $20 \pm 5\%$ for the first flight and $17 \pm 4\%$ for the second flight, making CO the second most abundant parent molecule in the coma. The derived production rate of atomic carbon is consistent with that expected from the photodissociation of carbon monoxide.

Atomic carbon is a common feature in the ultraviolet spectrum of many comets, yet its origin remains unclear. In comet West (1976 VI), the observed C I emission was consistent with a model of carbon as a daughter of CO which is vaporized from the nucleus as a parent molecule³. However, for comet Bradfield (1979X) the C I brightness and spatial distribution were not

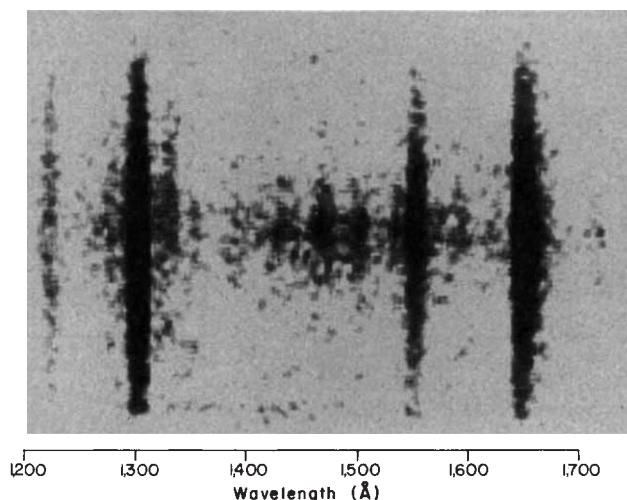


Fig. 1 A photographic representation of the raw counts from the spectrograph on NASA 21.093UG is shown for a 72.2-s exposure of comet Halley centred in the entrance slit of the spectrograph. The horizontal axis is the wavelength dispersion from $\sim 1,150$ to $1,800 \text{ \AA}$, and the vertical axis is along the 7.7 arc min slit with the sunward direction pointing down. The three brightest lines are O I $\lambda 1,304$, C I $\lambda 1,561$, and C I $\lambda 1,657$. H I $\lambda 1,216$ appears as a weak feature due to the heavy attenuation below $1,230 \text{ \AA}$ by a CaF_2 filter in front of the entrance slit.

consistent with models of carbon either as the daughter of the observed CO or as the granddaughter of CO_2 (ref. 4). The differences may reflect intrinsic compositional differences between comets, or deficiencies in the models used to interpret the observations. Festou⁵ has discussed this 'carbon puzzle' in the context of similar results on other comets observed by the International Ultraviolet Explorer (IUE) satellite.

The spectral range for the sounding rocket observations, $1,200\text{--}1,750 \text{ \AA}$ at $12.5 \text{ \AA}$ resolution, is well suited for studying the carbon chemistry of the coma as it includes C I $\lambda 1,657$, C I $\lambda 1,561$, C II $\lambda 1,335$, and the CO fourth positive system whose strongest bands are at $1,478$ and $1,510 \text{ \AA}$. While these observations augment the many ultraviolet observations of comet Halley made with IUE⁶, the sounding rocket payload is specially designed for the study of the emission profiles in the coma because the spectrograph provides 10 arc s spatial resolution along the 7.7 arc min slit. In addition, a higher sensitivity, needed because of the short observing time ($\sim 300 \text{ s}$) available with a sounding rocket experiment, is achieved using a photon-counting detector.

The faint object telescope (FOT) payload flown previously⁷ was modified for long-slit spectroscopy of extended emission objects. The basic structure of the FOT is a Dall-Kirkham telescope, a Rowland circle spectrograph and a slit-jaw camera. The spectrograph modifications included the use of an ellipsoidal grating to reduce astigmatism at the focal plane and a two-dimensional resistive anode detector. The first launch of the experiment, NASA 21.093UG, was on 26 February 1986 at 12:02 UT, 17 days after perihelion, when the heliocentric distance, r , was 0.70 AU and the geocentric distance, Δ , was 1.32 AU . The payload was recovered, refurbished, and flown again on 13 March 1986 at 11:20 UT when r was 0.90 AU and Δ was 0.98 AU . This flight, NASA 21.095UG, preceded the Giotto encounter by $\sim 13 \text{ h}$. Both launches were from the White Sands Missile Range, New Mexico (106.3° W , 32.4° N).

During both flights a central exposure was taken with the entrance slit (16 arc s wide by 7.7 arc min long) of the spectrograph centred on the brightest part of the coma and with the long axis of the slit oriented along the Sun-comet line. The first flight also included a tail exposure that was obtained with the slit centred 8 arc min away from the comet in the anti-sunward direction, again with the slit along the Sun-comet line.

Table 1 The brightness B and the derived column density N of the species in the central coma (10×16 arc s) observed in both flights

Species	NASA 21.093 UG: 26 February 1986, 12:07 UT			NASA 21.095 UG: 13 March 1986, 11:25 UT			IUE $B_{\pm}$ (R)
	B^* (R)	$g^{\dagger}$ (1 AU) (10^{-6} s^{-1})	N (10^{14} cm^{-2})	B^* (R)	$g^{\dagger}$ (1 AU) (10^{-6} s^{-1})	N (10^{14} cm^{-2})	
O I λ 1,304	$1,460 \pm 320$	3.7	1.9	280 ± 65	1.8	1.25	210 ± 40
C II λ 1,335	130 ± 30	50.0	0.013	25 ± 15	37.0	0.0054	—
CO λ 1,478	175 ± 50	0.24	3.5	65 ± 25	0.24	2.2	100 ± 60
CO λ 1,510	140 ± 40	0.18	3.8	60 ± 25	0.18	2.7	60 ± 40
C I λ 1,561	480 ± 110	6.0	0.39	150 ± 35	5.9	0.20	190 ± 50
C I λ 1,657	$1,600 \pm 350$	23.0	0.34	520 ± 115	22.0	0.19	745 ± 110

* The uncertainty is a combination of the 20% uncertainty in the absolute calibration of the instrument and the counting statistics.

† The solar fluorescence efficiencies (or g -factor) are based on earlier values¹⁴ which are adjusted for the heliocentric velocity of comet Halley and the solar flux (G. Rottman, personal communication) during the observation. The CO g -factors are from ref. 8 scaled to 1 AU and corrected by 0.5 to account for the variation of the solar flux.

‡ The average of IUE data from 12 and 13 March 1986; the CO brightness is from 13 March only.

A spectrogram of the central exposure taken during the first flight is represented as a two-dimensional photographic image in Fig. 1. The vertical axis depicts the spatial distribution of the emissions from the coma. The extended and brighter features in the spectrogram are H I λ 1,216, O I λ 1,304, C I λ 1,561, and C I λ 1,657, which have been observed in almost all comets studied in the far ultraviolet region¹. The spectra of the central coma (16×72 arc s centred on the nucleus) are shown in Fig. 2 for both flights. Other features identified in the spectra include the emissions from C II λ 1,335 and the CO fourth positive system. At least 11 bands of CO are clearly identified by comparison with a synthetic spectrum based on CO fluorescence of ultraviolet solar radiation⁸. While a few of these bands are seen in the IUE spectra of comet Halley⁶, the poor signal-to-noise ratio in the IUE spectra makes it difficult to extract reliable band intensity ratios from those data.

A tentative identification of O I λ 1,356 at a level of 60 R in the 26 February spectrum can be made. This intercombination line is not excited by solar fluorescence as the other emissions are, and suggests the presence of a region of electron excitation within the inner coma. Other indications for a collisional destruction mechanism within $\sim 10^4$ km of the nucleus besides solar photodissociation and photoionization are given by the IUE observations of CO₂⁺ during an outburst⁹ and by the unexpectedly high C⁺ abundance measured by Giotto¹⁰. An enhanced flux of keV electrons at 15,000 km measured by Vega 1 and Vega 2¹¹ and a maximum in the total ion current at 12,000 km as measured by Giotto¹² indicate that a region in the inner coma could exist where collisional excitation or destruction mechanisms are significant. Additional modelling is needed to verify this identification of O I λ 1,356.

The brightnesses of the strongest emission features for both flights are listed in Table 1, and are given for an area of 10×16 arc s centred on the nucleus of comet Halley. Table 1 also gives the derived column densities and the fluorescence efficiencies used to derive them. The IUE data in Table 1, given for comparison with NASA 21.095UG, are the average of observations made on 12 and 13 March 1986 at 22 UT on each day. The IUE data are reduced to an effective aperture of 10×15 arc s. These brightnesses are consistent within the uncertainty in the two measurements, which results from a combination of calibration errors and the different spatial resolution and tracking stability of the two instruments. Although the visual brightness of the comet varied by a factor of two over a period of a day during many of the IUE observations⁶, the O I and C I emissions should not vary as rapidly as their parents' lifetimes are longer than a day. Because the visual brightness was reasonably constant from 12 to 13 March, only a small effect due to the intrinsic variability is expected in comparing the two data sets.

As the composition of the comet's nucleus cannot be directly

inferred from the given brightnesses, modelling of the coma to fit the observed radial distributions of the emissions is performed to determine the production rates of the molecular species. A radial outflow model of CO with a production rate of $2.4 \times 10^{29} \text{ s}^{-1}$ for the first flight and $1 \times 10^{29} \text{ s}^{-1}$ for the second flight yields a good fit to the observed CO distributions (Fig. 3a). This

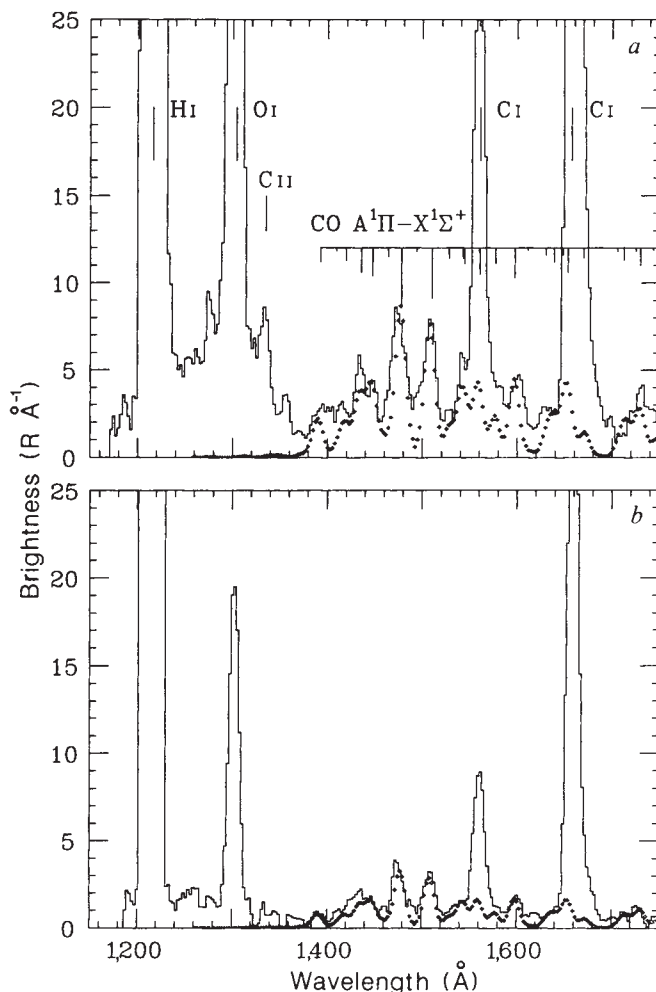


Fig. 2 The central coma spectrum from: *a*, the first flight on 26 February 1986; and *b*, the second flight on 13 March 1986. The synthetic CO spectrum (diamonds), convolved to our instrument resolution, is based on the g -factors of CO fluorescence of ultraviolet solar radiation given by Durrance⁸. The feature at 1,356 Å in *a* has been tentatively identified as O I λ 1,356 excited by electron collisions in the inner coma.

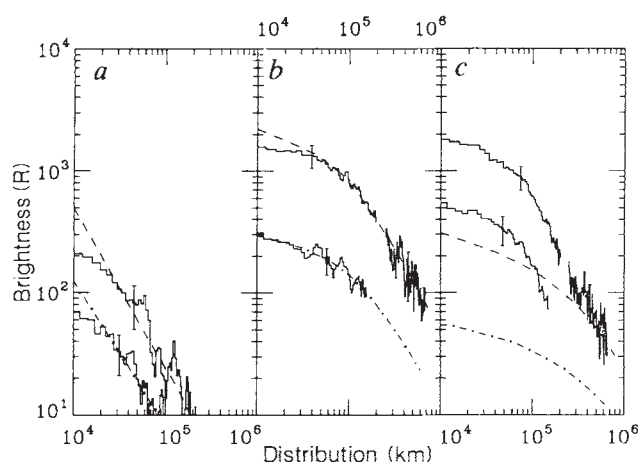


Fig. 3 The observed radial distribution of: *a*, the 1,478-Å CO band; *b*, O I 1,304; and *c*, C I 1,657 for both flights with the data from the first flight being the upper curve in each panel. The dashed lines are emission profiles, for comparison with the data from the first flight, convolved to our instrument resolution, from a radial outflow model with the production rate of $2.4 \times 10^{29} \text{ s}^{-1}$ for CO and $1.2 \times 10^{30} \text{ s}^{-1}$ for H₂O. The dot-dash lines, for comparison with the data from the second flight, are emission profiles from a model with the production rate of $1 \times 10^{29} \text{ s}^{-1}$ for CO and $6 \times 10^{29} \text{ s}^{-1}$ for H₂O.

strongly suggests that the CO is vaporized directly from the nucleus, but cannot exclude the possibility of a parent species which has a lifetime $< 1,000 \text{ s}$. A possible parent for CO is H₂CO, but it is improbable because a radial outflow model of H₂CO that reproduced the observed CO profile would require a production rate greater than the water production rate and would produce a detectable level of H₂ fluorescence in our spectral range. By fitting an outflow model with H₂O and CO parents to the observed oxygen distributions in Fig. 3*b*, a production rate for water of $1.2 \times 10^{30} \text{ s}^{-1}$ is derived for the first flight and $6 \times 10^{29} \text{ s}^{-1}$ for the second flight. The average water production rate derived from the OH (0,0) band brightness measured by IUE, using a vectorial model², is $5 \times 10^{29} \text{ s}^{-1}$ for comet Halley on 12 and 13 March 1986. The water production rate derived from the Giotto neutral mass spectrometer data¹² is $5.5 \times 10^{29} \text{ s}^{-1}$ with an estimated 50% uncertainty. Because of the uncertainties in the parameters (lifetime and velocity of each species) used in the model, the H₂O production rates derived from a coma model have at least a 30% uncertainty. Therefore, these water production rates are consistent within the uncertainties in the models, instrumental calibrations and the solar fluxes used in deriving the fluorescence efficiency for O I 1,304 (G. Rottman, personal communication). Note that because CO appears to be a parent molecule and is modelled with fewer parameters, the uncertainty in the derived CO production rate is smaller than the uncertainty in the H₂O production rate.

While data from the Giotto ion mass spectrometer gave an upper limit of 20% for the ratio of CO to H₂O abundance¹⁰, and a rough estimate from the IUE observations in March for this ratio is 10–20%⁶, the results from the rocket data give a relative abundance of CO to H₂O as $20 \pm 5\%$ for the first flight and $17 \pm 4\%$ for the second flight. The uncertainty in these ratios includes the errors from the absolute calibration of the instru-

ment and the fit of the models to the observed radial profiles. Krankowsky *et al.*¹² consider CO₂, NH₃ and CH₄ to be the second most abundant molecules in the coma; however, our results indicate that CO is the second most abundant parent molecule with a mixing ratio of $\sim 18\%$ relative to H₂O. For comparison, a 27% abundance of CO to H₂O was derived for comet West³, and 1% for comet Bradfield⁴.

While these data strongly suggest that the CO is vaporized directly from the nucleus, an unresolved difference remains between the observed radial distribution of carbon shown in Fig. 3*c* and the predictions of the CO outflow model. The data seem to require an additional source of carbon. An outflow model with a small additional source of carbon from the nucleus, at $\sim 2\%$ of the H₂O production rate, improves the fit to the observed carbon profile, but it is not adequate to identify firmly a direct source of carbon from the nucleus. An alternative evaluation of the atomic carbon production rate can be obtained if the total flux in a C I emission multiplet is known. Although the present experiment did not measure the total flux, a reliable estimate can be obtained from the observed radial profiles because the measurements in the tailward exposure on the first flight extend to $7 \times 10^5 \text{ km}$. Circular symmetry of the carbon emission, necessary for this derivation, is a safe assumption as the observed carbon emissions are symmetrical on the sunward and anti-sunward sides of the nucleus. The derived carbon production rate, Q_C , is $4.3 \times 10^{28} \text{ s}^{-1}$ for the first flight. As the carbon scale length is $\sim 2.5 \times 10^6 \text{ km}$ at 0.70 AU, this production rate is only a lower limit. Extrapolating the observed carbon radial distribution to $3 \times 10^6 \text{ km}$ yields $Q_C = 6.4 \times 10^{28} \text{ s}^{-1}$, while extrapolation to $1.5 \times 10^7 \text{ km}$ gives $Q_C = 6.6 \times 10^{28} \text{ s}^{-1}$. Thus, a reasonable estimate for the total carbon production rate for the first flight is $7 \times 10^{28} \text{ s}^{-1}$, which gives a ratio of Q_{CO}/Q_C of 3.4 ± 1.2 . Because our derived carbon production rate is a lower limit, we consider this ratio to be consistent with the expected ratio of 2.1, assuming that C is produced only through the photodissociation of CO (ref. 13).

The apparent contradiction between the need for an additional carbon source and the result that the Q_{CO}/Q_C ratio is consistent with photodissociation of CO may be resolved by the inclusion of an electron impact source in the models. Evidence for such a source, including the O I 1,356 emission in Fig. 2, has been cited above. If electron impact enhanced the dissociation of CO in the inner coma, without destroying a significant fraction of the total CO, then the radial distribution of C would exhibit a steeper slope in agreement with the observations, while the radial distribution of CO and the Q_{CO}/Q_C ratio would not be significantly altered. Quantitative modelling using the *in situ* plasma measurements^{10,11} is needed to evaluate the possible importance of this mechanism. In addition, improved coma models, such as the vectorial model², or models based on the *in situ* measured abundances of CO₂, CH₄, and other carbon-bearing molecules may also provide better agreement with the observed radial distributions of the carbon emissions.

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Far-ultraviolet spectral images of comet Halley from sounding rockets

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Wide-field ultraviolet imagery of comets provides a useful means of determining such fundamental parameters as production rates, lifetimes and outflow velocities of daughter photodissociation products in the coma. Far-ultraviolet imagery has been used to measure the production rates and outflow characteristics of hydrogen for comets Bennett¹, Kohoutek^{2,3} and West^{4,5}, for C and O in comet Kohoutek⁶, and for C, S and CS in comet West⁷. Ultraviolet spectroscopy has also revealed the presence of C⁺ ions and CO molecules⁸ in comet West. Other comets have been observed spectroscopically in the ultraviolet from the International Ultraviolet Explorer (IUE) spacecraft⁹. Here we present far-ultraviolet images of comet Halley obtained from sounding rockets launched from White Sands Missile Range, New Mexico, on 24 February and 13 March 1986. We gained direct electrographic images of the hydrogen coma of the comet at the Lyman- α wavelength (1,216 Å), and objective spectra containing images of the coma at the oxygen (1,304 Å), carbon (1,561 and 1,657 Å) and sulphur (1,814 Å) resonance multiplets. Analysis of the Lyman- α images yields hydrogen atom production rates of $1.9 \times 10^{30} \text{ s}^{-1}$ and $1.4 \times 10^{30} \text{ s}^{-1}$ for the two observations. Images of oxygen, carbon and sulphur emissions obtained with the objective grating spectrograph are presented for the first set of observations and preliminary production rates are derived for these elements.

Preliminary results of two far-ultraviolet rocket imaging experiments are reported here. The first rocket (NASA 36.010 DL), was launched as soon after perihelion as practical to observe the comet at high luminosity. The payload was refurbished at the Naval Research Laboratory between launches. The second rocket (NASA 36.017 DL) was launched just before the Giotto spacecraft flyby of comet Halley. Both experiments were coordinated with rocket observations of comet Halley by Johns Hopkins University experimenters (accompanying paper)¹⁰. The relative solar and cometary positions at the time of the two flights are given in Table 1.

The rocket payload instruments were similar to those used previously in sounding rocket observations of comet Kohoutek². The payload contained a direct-imaging camera covering the wavelength range 1,050–1,600 Å (which includes the hydrogen Lyman- α line at 1,216 Å), and an objective grating spectrograph covering the range 1,230–2,000 Å. Also included was a Lyman- α airglow calibration photometer using a photoionization chamber filled with nitric oxide as a detector.

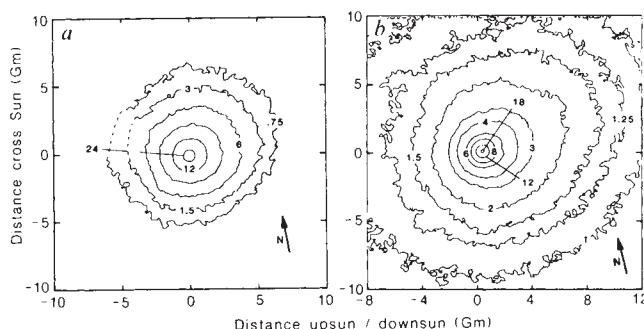


Fig. 1 Isophote contours (kR) of Lyman- α images of comet Halley. Exposure times are 2.5 s for the 24 February (a) and 9.5 s for the 13 March (b) data. The distance scales are the same and the Sun is to the left. The dotted contours on the 24 February plot are reconstructions of isophotes marred by static discharges on the film. Backgrounds due to the terrestrial Lyman- α airglow of an effective 7 kR for the first observation and 6.5 kR for the second have been subtracted.

The direct imaging camera had a 75-mm focal length, $f/1$ Schmidt optical system (LiF corrector plate) and a KBr photocathode; it was identical to Lyman- α cameras used in previous rocket observations of comets Kohoutek and West²⁻⁴. The camera field of view was 20° in diameter with an angular resolution of ~ 3 arc min.

The objective grating spectrograph was based on an electrographic Schmidt camera with a focal length of 150 mm, $f/1.5$ focal ratio, CaF₂ corrector plate and CsI photocathode. A objective grating with 1,200 lines per mm was used that gave a spectral dispersion at the photocathode of 30 Å mm⁻¹. The spectrograph had a 12° square field of view and angular resolution of ~ 1 arc min. The corresponding point-source spectral resolution was ~ 1 Å. This camera was much more sensitive than that used in the previous objective spectroscopy of comet Kohoutek because it incorporated a microchannel plate (MCP) intensification stage^{11,12}. The MCP was operated at a gain of ~ 100 , giving a corresponding increase in effective diffuse-source sensitivity.

Laboratory calibrations consisted of measurements of relative wavelength sensitivity in the 1,150–1,850 Å range combined with absolute measurements of a monochromatic 1,304 Å spot source of diameter 0.27°. For the Lyman- α camera, in-flight calibration was obtained from the ion chamber, and a relative calibration between the two flights was obtained from simulated aperture photometry of the image of the star π Capricornis, which in both cases was in the field of view. Laboratory and in-flight calibrations agreed closely, and the estimated absolute accuracy of the Lyman- α measurements is $\pm 20\%$. The reduction of the laboratory calibration data for the spectrograph data is complex and has not been completed; consequently an in-flight calibration based on the spectrum of τ Capricornis, which was adjacent to the comet spectrum, was used. The observed τ Cap spectrum was calibrated using the OAO-2 photometer measurements at 1,910 Å (ref. 13), to which was normalized an OAO-2 spectrum of the star ζ Draconis¹⁴ which has the same spectral type and colour (B6 III, (B–V) = –0.12). The wavelength scale in the stellar spectrum was based on an IUE spectrum of HD 182255 (ref. 15), that also has the same spectral type and reddening. The TD-1 spectrophotometry of τ Capricornis¹⁶ is available down to 1,380 Å, but is about 15% below the OAO-2 measurement at 1,910 Å. The absolute accuracy of the spectral data is $\sim \pm 35\%$, including the uncertainty in the comparison star spectrophotometry, but the relative (wavelength) accuracy is $\sim \pm 10\%$.

The flight and calibration exposures were scanned and digitized using a microdensitometer at the University of Texas, Austin, using a 16 $\times$ 16 μm aperture on a 2048 $\times$ 1536 array. The measurements were then summed on a 2 $\times$ 2 basis to form a

Table 1 Observation parameters

	36.010 (24 February 1986)	36.017 (13 March 1986)
Time of launch	11:53:30 UT	10:54:00 UT
Apogee (km)	312	312
Comet zenith angle (apogee)	91°	87°
Solar zenith angle (apogee)	112°	120°
Comet–Sun distance (AU)	0.672	0.895
Comet–Earth distance (AU)	1.356	0.975
Comet–Sun velocity (km s ⁻¹)	17.97	25.57
Comet–Earth velocity (km s ⁻¹)	–32.11	–43.32
Sun–comet–Earth angle	44.0°	64.1°
Sun–Earth–comet angle	28.2°	54.1°

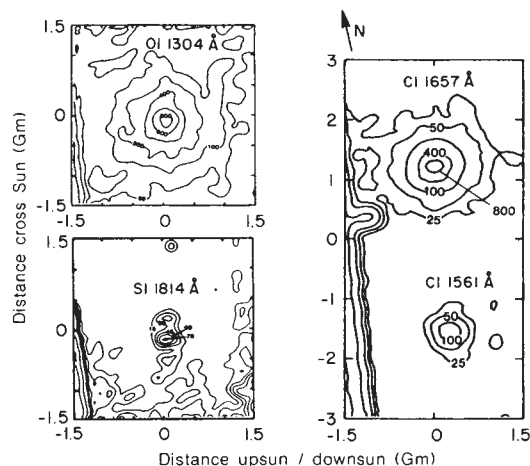


Fig. 2 Brightness in rayleighs of the objective spectral images of C, O and S resonance multiplets seen during the flight of 24 February. The images have been compressed in the spectral direction to correct for the non-isotropic plate scale of an objective spectrograph. The brightness contours have been oriented so that the Sun is positioned to the left. A background derived from an average in the vicinity of each feature was subtracted from all four images. The vertical streaks on the left are from the spectrum of τ Capricornus which was used for calibration. The right sides show a small amount of contamination from another stellar spectrum, and contours from zero order star images are seen.

1,024 × 768 array for final analysis; the resultant sampling was 2–3 pixels per resolution element. Nonlinearities are small in the electrographic process below a film density of ~ 4.0 , and were avoided by using data from shorter exposures for the brighter features.

Images of the Lyman- α coma of comet Halley were obtained on both flights with 0.5, 2.5, 9.5 and 29.5 s exposure times. Contour plots of the Lyman- α isophotes from images obtained on the two periods of observation are given in Fig. 1. Only the 0.5 and 2.5 s exposures from the first flight could be used because of instrumental difficulties.

The intensity contours show approximately circular isophotes within 2 Gm of the nucleus but also show noticeable elongation away from the Sun at increasing distance. The surface brightness during the February observations was higher, but the outer isophotes of the March image are better developed due to the longer exposure time and decreased Earth-comet distance.

In both rocket flights, objective spectral images of the comet were obtained with exposure times of 2.5, 9.5 and 29.5 s. Grayscale reproductions of images from the first flight have been published¹⁷. Contours of the surface brightness of the O 1,304 Å, C 1,561 Å and 1,657 Å, and S 1,814 Å multiplets are shown in Fig. 2. Because the inner regions of the O 1,304 Å and C 1,657 Å images were saturated on the film for exposure times > 2.5 s, the isophote contours shown for these features are a combination of images with long and short exposure times. Isophotes for contours > 100 R were taken from 2.5 s exposures for both features. The outer isophotes of the 1,304 Å image were taken from a 9.5 s exposure and those of the 1,657 Å images were taken from a sum of six 29.5 s images. The C 1,561 Å and S 1,814 Å isophotes were also taken from the sum of six images.

The production rates of C, O, S and H were calculated using a simple radial-outflow model similar to that used previously to analyse hydrogen imagery^{2,4}. Atoms are assumed to expand outwards isotropically from a negligibly small source region. The atoms are ultimately lost to photoionization or charge exchange with the solar wind; however, in the inner regions losses can be ignored and the column density of atoms at projected distance ρ from the nucleus can be related to the production rate Q , and mean outflow velocity $\bar{v}$, by

$$N(\rho) = Q/(4\rho\bar{v})$$

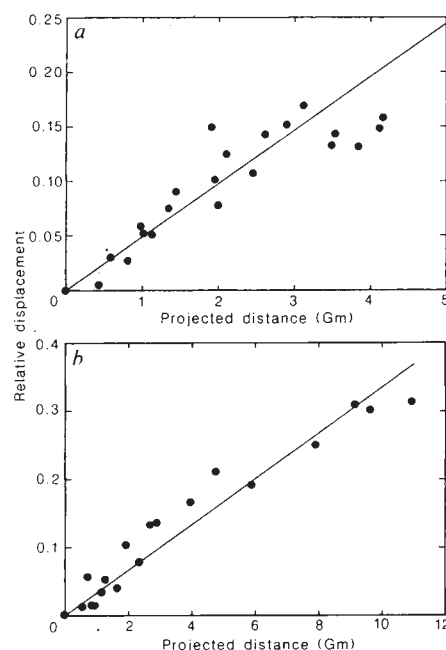


Fig. 3 Relative shift of isophote centre, S/ρ , versus ρ , the projected radius of the isophote. *a*, 24 February; *b*, 13 March. The lines show the predictions for a simple radial outflow model with $\bar{v} = 8 \text{ km s}^{-1}$ and the acceleration due to solar Lyman- α radiation pressure produced by a solar flux of $2.3 \times 10^{11} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$ at 1 AU.

The brightness of the comet in rayleighs at ρ is:

$$B(\rho) = 10^{-6} N(\rho) (g_0/R^2)$$

where g_0 is the g -factor, or scattering probability per (atom-second) at 1 AU, and R is the Sun-comet distance in AU. Thus, if the mean outflow velocity is known, the production rate can be determined by measuring the brightness at a distance ρ that is large compared to the instrument resolution but small compared to the scale length of the loss processes.

The radial outflow velocity for heavy constituents is typically 1 km s^{-1} , and is the value adopted here for C, O and S to be consistent with previous calculations. For hydrogen, because of its small mass, a high outflow velocity is imparted during dissociation which can range from a few km s^{-1} to over 20 km s^{-1} depending on the dissociation channel. A mean outflow velocity for H can be determined by measuring the shift of the inner Lyman- α isophotes caused by solar radiation pressure^{2,4}. The shell of hydrogen emitted at time t before the observation will have been accelerated downsun a distance $S = at^2/2$, where a is the acceleration due to radiation pressure. Because of limb brightening most of the contribution to the observed radiation from a given shell is from a ring of projected distance equal to the radius $\rho = \bar{v}t$. A plot of relative isophote shift versus radius then gives the velocity (Fig. 3). The prediction for $\bar{v} = 8 \text{ km s}^{-1}$ is shown; this is in good agreement with results obtained for comets Bennett, Kohoutek and West. In spite of the many simplifying assumptions, this model tends to give production rates similar to those obtained from more realistic computations.

Production rates derived for the four species on 24 February and for H on 13 March are summarized in Table 2. The formula above was used, taking the average brightness of the coma at the radius indicated in Table 2 and assuming an outflow velocity of 1 or 8 km s^{-1} as appropriate. The sulphur brightness listed is the sum from the two bright lines that are resolved, and is the least reliable because of the poor signal-to-noise ratio for this multiplet.

The Lyman- α observations on 13 March give production rates of H_2O , (assuming all H comes from water), of $7 \times 10^{29} \text{ s}^{-1}$, in

Table 2 Production rates

Species	ρ (Gm)	$B(\rho)$ (R)	g_0 (1 AU) (s ⁻¹)	Outflow velocity (km s ⁻¹)	Q (s ⁻¹)
H (24 Feb)	2.0	7,850	1.22×10^{-3}	8	1.9×10^{30}
O	0.2	406	3.7×10^{-6}	1	5.9×10^{29}
C	0.2	535	2.3×10^{-5}	1	8.4×10^{28}
S	0.12	51	5.9×10^{-5}	1	2.4×10^{27}
H (13 Mar)	1.0	6,680	1.22×10^{-3}	8	1.4×10^{30}

Values for g_0 for C and O from ref. 21, g_0 for H from ref. 4, g_0 for S from ref. 7. The 1,657 Å emission was used for the C production rate.

good agreement with results from optical and mass spectrometers on the Vega^{18,19} and Giotto²⁰ spacecraft. The 24 February observations given an H/O ratio of >3, perhaps indicating that not all the hydrogen is produced from water (uncertainties could be as large as 50% for O). Our C production rate is consistent with results from the other rocket observations¹⁰, and indicates that gaseous carbon is relatively scarce in this comet. Our C/S ratio is not very different from the value of 22 given by ref. 7. The cosmic ratio is 21.

Further reduction of the data is in progress. Future analyses of the spectral images will involve the use of more complex models, and comparisons will be made with Lyman- α observations from the Suisei, IUE, Dynamics Explorer and Pioneer Venus spacecraft.

Post-perihelion observations of water in comet Halley

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Our previous pre-perihelion observations¹ of comet Halley from the NASA Kuiper Airborne Observatory (KAO) resulted in the first definite detection of water vapour in a comet. This success allowed us to carry out an ambitious post-perihelion KAO observing programme which significantly extended the scope of our Halley water investigation. The spectral bandwidth of the post-perihelion data included essentially all of the ν_3 band. Fifteen spectral lines of water were detected, including three previously undetected lines in the ν_3 band ($\lambda \approx 2.65 \mu\text{m}$) and three lines in the (011–010) band. The observed relative intensities illustrate that water in the cometary coma is rotationally relaxed, as predicted by recent nonthermal-equilibrium models^{2,3}. An *ortho/para* ratio of ~ 3 is consistent with both pre- and post-perihelion data. The water spatial brightness profile can be fit by that of a parent molecule, and a significant sunward–tailward brightness asymmetry was observed which suggests ejection primarily into the sunlit hemisphere. Comet Halley exhibited marked temporal activity on timescales as short as 2 h. The largest water production rate measured was $2.3 \times 10^{30} \text{ mol s}^{-1}$ on 24:18 UT March 1986, which is about an order of magnitude (or more) larger than the production rates we measured pre-perihelion at the same heliocen-

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tric distance ($\sim 1 \text{ AU}$), indicating a large pre- to post-perihelion asymmetry in gas production.

Spectra were obtained on 20, 22, 24 and 26 March 1986 using the University of Arizona high-resolution Fourier-transform spectrometer (FTS). The heliocentric distance (R) was $\sim 1 \text{ AU}$ for all dates while the geocentric distance (Δ) decreased from 0.8 to 0.6 AU during our investigation. Observations from airplane altitudes ($\sim 12 \text{ km}$) and large cometary geocentric radial velocities ($\dot{\Delta} = -43 \text{ km s}^{-1}$ for the period of our observations) were required to obtain good atmospheric transmittance at the positions of cometary H_2O emission lines. Comet Halley was in the airplane's observing 'window' for up to 2.5 h during our flights. For monitoring the temporal variability of the comet, at least one spectrum was taken each flight through a narrow filter ($\Delta\lambda \leq 0.06 \mu\text{m}$) using the relatively modest spectral resolution of $\sim 1.4 \text{ cm}^{-1}$. Approximately 15 min were required to obtain these data when our aperture ($\sim 41 \text{ arc s}$ diameter) was centred on the nucleus. Spatial mapping was accomplished using the same instrumental setup, but with the aperture offset by known amounts from the centre of brightness of the comet. On 26 March we observed comet Halley at $\sim 0.75 \text{ cm}^{-1}$ resolution through a much wider filter ($1.9 \leq \lambda \leq 2.7 \mu\text{m}$), which transmits essentially the entire ν_3 band of H_2O . On 22 March we used the narrow filter at $\sim 0.014 \text{ cm}^{-1}$ resolution ($\Delta v = 1.2 \text{ km s}^{-1}$), but these data will be discussed elsewhere⁵.

Figure 1 shows the spectra acquired through our wide band-pass filter: Fig. 1a is a spectrum of the Moon showing the atmospheric transmittance, and Fig. 1b is the cometary emission spectrum. Figure 1a is characteristic of an H_2O absorption line spectrum in thermal equilibrium at $\sim 216 \text{ K}$ and shows significant absorption for rotational levels up to $J = 8$ ($J \equiv$ total angular momentum quantum number). In contrast, the cometary spectrum is heavily weighted towards low J , with all detectable emission originating from transitions having $J \leq 3$ as the lower level.

The intensities for all observed H_2O lines are given in Table 1. At least six new H_2O lines were detected post-perihelion that were not observed pre-perihelion. Most of these were not observed pre-perihelion simply because the comet had significantly stronger emissions post-perihelion (that is, $Q_{\text{H}_2\text{O}}$ was

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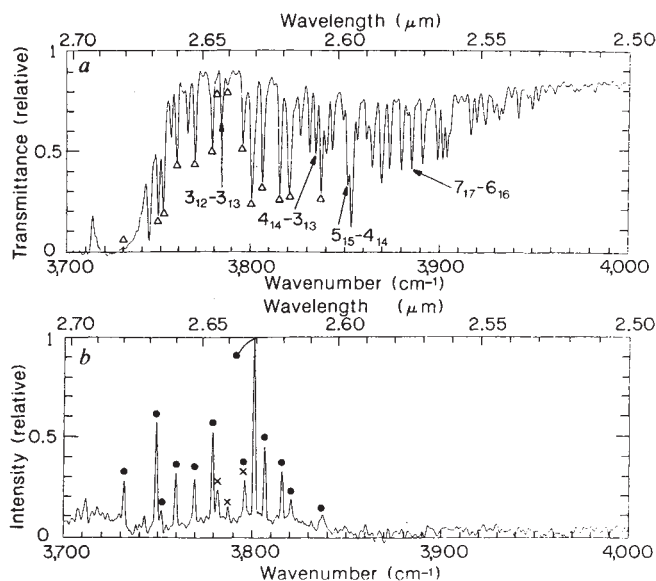


Fig. 1 *a*, A spectrum of the Moon. *b*, The spectrum of comet Halley. Both spectra, taken on 26 March 1986, are apodized to a resolution of 1.5 cm^{-1} , and are uncorrected for atmospheric transmission and instrumental response. For the Halley spectrum, $R = 1.096 \text{ AU}$ and $\Delta = 0.646 \text{ AU}$. The absorption lines in the lunar spectrum are due to H_2O and CO_2 in the Earth's atmosphere. Terrestrial H_2O lines corresponding to observed cometary lines are indicated (Δ). The relative strengths of the H_2O lines are characteristic of thermal equilibrium with $T \sim 216 \text{ K}$. Four H_2O lines whose lower states are the upper states for rotational transitions which have been suggested as probes of H_2O in comets are also identified. These lines are not present in the cometary spectrum. The cometary spectrum is due primarily to molecular line emission produced in solar infrared fluorescence (a little cometary continuum may also have been detected). Most lines are in the (001-000) vibrational band ($\bullet$), but three lines can be seen in the (011-101) band ($\times$). Note the contrast between the LTE (low thermal emission) terrestrial transmittance spectrum (*a*), which shows significant H_2O absorption for $J \leq 8$, and the non-LTE cometary spectrum (*b*) which sharply cuts off with $J \leq 3$ (J = lower state total angular momentum quantum number).

much larger) and/or the lines were not transmitted by our pre-perihelion filter. There are three lines in our spectra which belong to the (011-010) 'hot' band of H_2O . Our calculations of the g -factors for this band yield predicted intensities which match the observed values. One of these lines is so strong that it should have been detected in the pre-perihelion spectrum given similar excitation conditions pre- and post-perihelion. The absence of this line pre-perihelion remains to be explained.

The lines originating from levels other than the lowest *ortho* and *para* states (1_{01} and 0_{00} , respectively) have become relatively stronger post-perihelion. This behaviour seems consistent with the hypothesis that larger post-perihelion water production rates cause the pure rotational transitions (which allow relaxation of the H_2O molecules to the lowest states) to become extremely optically thick, thereby 'pumping up' the higher J levels. We note, however, that this mechanism apparently still does not significantly populate levels with $J > 3$. In fact, assuming that the $4_{04}-3_{03}$ line is pumped only from 3_{03} , we use the measured post-perihelion intensity of this line to derive a relative (to the total) population of $4.3 \pm 1\%$ in the 3_{03} level, confirming our previous conclusion¹ that the population in 3_{03} must be small.

Figure 1*a* shows the positions of several strong terrestrial H_2O lines in the ν_3 band whose lower states are the upper states of some rotational transitions which have been suggested as probes of cometary H_2O ($6_{16}-5_{23}$ at 22 GHz, $3_{13}-2_{20}$ at 183 GHz, and $4_{14}-3_{21}$ at 380 GHz). The absence of cometary infrared fluorescence (Fig. 1*b*) in these lines ($3_{12}-3_{13}$, $4_{14}-3_{13}$, $5_{15}-4_{14}$ and

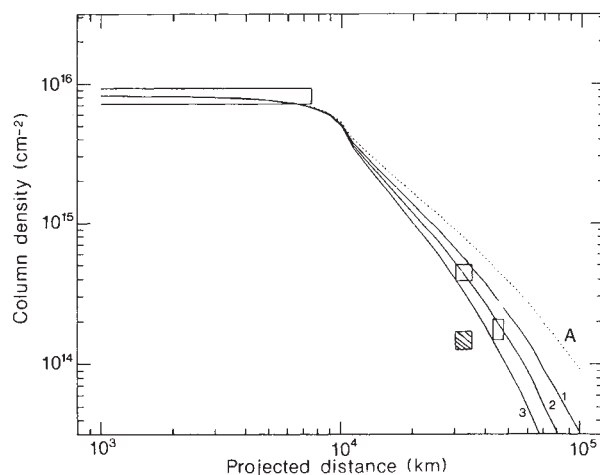


Fig. 2 Assuming that the H_2O emission in the ν_3 band is proportional to the H_2O column density, the boxes represent the observed H_2O spatial brightness distribution. The width of each box is the estimated pointing uncertainty for each observation and the height is the error in the measured relative intensities. The largest component of this error ($\sim 10\%$) is the uncertainty in deriving the relative ν_3 band emission for the nucleus pointings versus the offset pointings (the excitation changes with position in the coma). Two of the offset pointings were towards the Sun while the hatch box represents a tailward pointing. A significant sunward-tailward brightness asymmetry was measured, so only the sunward points are used to determine the H_2O scale-length. Although $Q_{\text{H}_2\text{O}}$ was varying significantly over the time required for molecules to flow from the nucleus to the offset points, we have ignored this effect. Comparison of KAO and IUE data suggest that $Q_{\text{H}_2\text{O}}$ was increasing before our observations, which implies that the scale-length derived from our data is a lower limit to the actual value. The curves represent steady-state radial outflow models which have been convolved with a 41 arc s diameter aperture. Scale-lengths: model A, $8.2 \times 10^4 \text{ km}$; model 1, $4.4 \times 10^4 \text{ km}$; model 2, $3.2 \times 10^4 \text{ km}$; model 3, $2.4 \times 10^4 \text{ km}$.

$7_{17}-6_{16}$) suggests that the rotational levels 3_{13} , 4_{14} and 6_{16} are not significantly populated. This may explain some past failures to detect H_2O rotational lines in comets. We observe ν_3 band transitions which pump the levels 3_{13} and 4_{14} of the ground vibrational state, but the equilibrium populations achieved by the pumps are insufficient to produce detectable infrared fluorescence from those levels. If the rotational transitions $3_{13}-2_{02}$ and $4_{14}-3_{03}$ are optically thin, then the emission in two of the above rotational transitions can be estimated directly from the infrared emission rate. We calculate aperture-averaged intensities of $2.3 \times 10^{-16} \text{ W cm}^{-2} \text{ sr}^{-1}$ for the 183 GHz line and $2.4 \times 10^{-16} \text{ W cm}^{-2} \text{ sr}^{-1}$ for the 380 GHz line. Using a Haser model, these intensities can be used to predict the fluxes in a diffraction-limited (for a 0.9-m telescope) aperture. We obtain 28 Jy and 10 Jy for the 183 GHz and 380 GHz lines, respectively, which are below detection limits. However, these predicted fluxes should be considered as lower limits to the actual values because optically thin conditions were assumed and other possible pumping mechanisms were not considered.

The ratio of *ortho*- H_2O to *para*- H_2O in comets may be indicative of the temperature of the cometary ice¹. Our post-perihelion data give an *ortho/para* ratio of 3.23 ± 0.37 , compared with our revised pre-perihelion value of 2.73 ± 0.17 (the pre-perihelion value has been revised slightly due to a reassessment of the atmospheric transmittances). Within the present $\pm 2\sigma$ statistical error limits, both the pre- and post-perihelion data are in agreement that the *ortho/para* ratio in comet Halley is ~ 3 . More precise determination of the atmospheric transmittances is in progress, and should lead to reduced error estimates for the cometary line intensities, and for the *ortho/para* ratio.

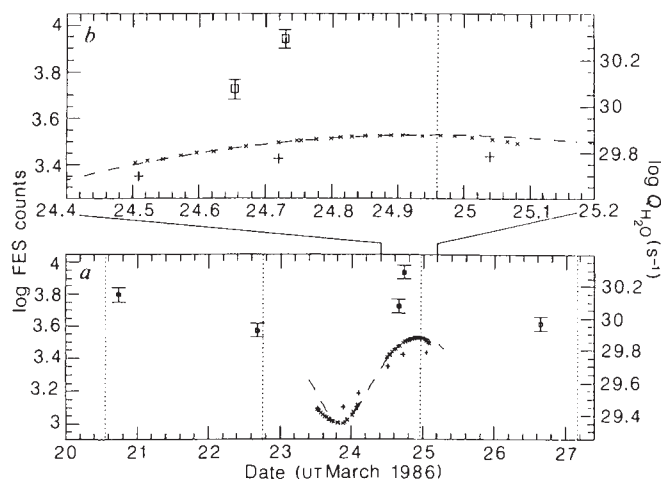


Fig. 3 *a*, Water production rates (using model A parameters) derived for all KAO observations ($\square$). The error bars are determined solely by our estimate of how pointing inaccuracies can affect our individual observations. The statistical error in the measurements is negligible by comparison. IUE data for 23 and 24 March are also shown. The IUE water production rates (+) are derived from measurements of OH. The integrated visual intensities measured by the IUE fine error sensor (FES) are also given ($\times$). The dashed curve is a sinusoid of period 2.2 days and gives a fairly good match to the FES curve. Propagating this sinusoid forward and backward in time would yield peaks in activity at the positions of the dotted vertical lines. *b*, An expansion of *a* during the time of simultaneous KAO and IUE observations. The temporal variability of the KAO data seems to be substantially larger than that of the IUE data for the time around 24:17 March UT.

Spatial information on the H_2O emission is required for determining the H_2O scale-length. On 24 March 1986 we made observations with sunward and tailward (approximately) offsets of 65 arcs (3.3×10^4 km at the comet) relative to the nucleus, and bracketed these by two exposures centred on the nucleus. On 26 March we made an observation centred on the nucleus and one offset by 97 arcs sunward.

As illustrated in Fig. 2, a significant sunward–tailward brightness asymmetry is observed, which suggests ejection of H_2O primarily from the sunlit hemisphere. The Giotto multi-colour camera⁶ pictures of Halley showed an asymmetry in nucleus activity directly, and our data demonstrate that the asymmetry persists far from the nucleus. We will ignore this asymmetry and derive an H_2O scale-length using only the sunward brightness profile and a spherically symmetric model.

Assuming that the measured brightnesses are linearly proportional to the H_2O column density, we show predicted brightness profiles for several scale-lengths in Fig. 2. The observed H_2O spatial brightness distribution is consistent with that of a parent molecule. The scale-length which best matches our data is $\sim 3.2 \times 10^4$ km (model 2, $R = 1$ AU). Taking 1 km s^{-1} as the nominal outflow velocity implies an H_2O lifetime which is 2.8 times shorter than the photochemical value at solar maximum⁷. Our very high resolution data⁵ are consistent with an H_2O velocity of $\sim 1.4 \text{ km s}^{-1}$, indicating that the H_2O lifetime could be shorter still.

The scale-length derived from the Giotto neutral mass spectrometer⁸ is given approximately by model 1 and is marginally consistent with our data. However, our data are not consistent with model A, which is based on the calculated photochemical lifetime. The short H_2O lifetime implied by both the Giotto and KAO observations is surprising because the Halley apparition occurred near the time of solar minimum when the H_2O lifetime should have its largest value (even larger than that of model A).

Temporal variation in $Q_{\text{H}_2\text{O}}$ may explain why our data give short apparent scale-lengths. For a pointing centred on the

Table 1 Intensities of H_2O lines

Transition*	Rest frequency† (cm^{-1})	Intensities‡
000–101 (001–000)	3,732.135	154 ± 78
111–110 (001–000)	3,749.330	114 ± 23
220–221 (001–000)	3,752.213	19 ± 4
110–111 (001–000)	3,759.845	38 ± 6
211–212 (001–000)	3,769.890	37 ± 8
101–000 (001–000)	3,779.494	42 ± 3
202–101 (011–010)	3,782.179	14 ± 1
211–110 (011–010)	3,787.666	6 ± 1
313–212 (011–010)	3,796.083	7 ± 1
212–111 (001–000)	3,796.440	18 ± 1
202–101 (001–000)	3,801.420	100 ± 5
211–110 (001–000)	3,807.015	44 ± 3
313–212 (001–000)	3,816.092	30 ± 3
303–202 (001–000)	3,820.739	12 ± 2
404–303 (001–000)	3,837.870	12 ± 3

* The quantum numbers of the upper and lower states $J'K'_aK'_c - J''K''_aK''_c$, and the vibrational band designation ($v'_1v'_2v'_3 - v''_1v''_2v''_3$) are given.

† From ref. 12.

‡ Relative intensities are listed. The integrated line intensity for the 202–101 (001–000) transition was $9.24 (\pm 0.09) \times 10^{-10} \text{ W cm}^{-2} \text{ sr}^{-1}$ on 24:18 UT March 1986. The estimated ν_3 band intensity in this case was $1.18 (\pm 0.11) \times 10^{-8} \text{ W cm}^{-2} \text{ sr}^{-1}$. The quoted errors are 1σ statistical errors plus our estimate of the uncertainties in the atmospheric transmittances.

nucleus, $\sim 70\%$ of the observed H_2O molecules are in a sphere whose radius is equal to the projected size of our aperture radius at the comet (1.04×10^4 km). If the molecules are moving at $\sim 1 \text{ km s}^{-1}$, then it takes ~ 2.9 h for an H_2O molecule to move from the nucleus to the edge of this sphere. Thus, the nuclear pointings mainly represent an integral of $Q_{\text{H}_2\text{O}}$ over the previous 3 h. As ~ 9 h and ~ 13 h are required for H_2O molecules to reach the two offset positions. These pointings represent an integral of $Q_{\text{H}_2\text{O}}$ over a period beginning much earlier than the time sampled by the nucleus pointing. Analysis of KAO and International Ultraviolet Explorer (IUE) data⁹ suggests that $Q_{\text{H}_2\text{O}}$ was rising during the 12 h preceding our observations. If this is true, then the scale-length derived from our data will be a lower limit to the actual value.

Using a nominal H_2O velocity of 1 km s^{-1} , and the scale-length given by model 2 of Fig. 2, we obtain a water production rate of $2.3 \times 10^{30} \text{ mol s}^{-1}$ for the observation with the largest H_2O emission (24:18 UT March). Below, we compare our data with water production rates derived from IUE measurements of OH, which also assume isotropic production and use the H_2O parameters of model A ($v = 1 \text{ km s}^{-1}$). For this model, the KAO data imply a production rate of $1.9 \times 10^{30} \text{ mol s}^{-1}$.

The model A water production rates derived from our observations are plotted in Fig. 3. Simultaneous IUE observations⁹ were made on 24 March, and the derived H_2O production rates are plotted in Fig. 3, along with the count rate from the IUE fine error sensor (FES; this is an acquisition camera being employed essentially as a photometer during these Halley observations).

The FES data display an apparent harmonicity with a period which is tantalizingly close to the value suggested by others¹⁰ as the rotation period of the comet (2.2 days). Assuming that there is a fundamental period of 2.2 days, we have indicated by the dotted vertical lines in Fig. 3 where the peaks in cometary activity should occur. The post-perihelion KAO data show clearly that the cometary activity cannot be described so simply; a peak in activity would be expected on 22 March, while the data show no evidence for this. Similar departures from simple periodicity were observed for IUE observations made during the week of the spacecraft encounters¹¹.

All the data illustrate the extremely dynamical behaviour of comet Halley, but the KAO H_2O data show the largest variability. At a time when the H_2O brightness increased by $\sim 64\%$, the FES counts and OH brightness increased by only $\leq 6\%$ (see Fig. 3b). These results indicate that the H_2O line brightnesses are very sensitive remote indicators of nucleus activity.

The post-perihelion KAO production rates seem to be systematically larger than those derived from the IUE measurements (a factor of ~ 2). However, the discrepancy between the KAO and IUE numbers may be due in part to absolute calibration uncertainties. Based on a comparison of the signal-to-noise ratio achieved on our data, the nominal instrument sensitivity, and the absolute fluxes produced in our calibration process, we expect that the KAO absolute intensities (from which we derive $Q_{\text{H}_2\text{O}}$) should be accurate to within $\pm 50\%$. Because the IUE absolute calibration uncertainty is $\pm 10\%$, the peak water production rates derived from the IUE measurements are actually consistent (at the 1σ level) with the average KAO value.

Besides absolute calibration difficulties, several other factors must be considered when comparing the KAO and IUE results. Both the KAO and IUE data have been reduced assuming that all cometary emissions are optically thin. The aperture-averaged optical depths for the strongest lines are ~ 1 for both the KAO and IUE observations, so this assumption is not valid. However, a more important consideration is the effect of temporal activity on the derived $Q_{\text{H}_2\text{O}}$. Our data indicate that $Q_{\text{H}_2\text{O}}$ derived from the KAO observations is more sensitive to temporal activity than $Q_{\text{H}_2\text{O}}$ derived from the IUE observations. Until time-dependent effects are treated properly, strict comparisons of water production rates derived from the two sets of data are not valid. Similar comments should apply to water production rates derived from radio OH observations.

Finally, we note that both our pre- and post-perihelion KAO observations were made when the comet was at a heliocentric distance of ~ 1 AU. A significant pre- to post-perihelion asymmetry in the water production rate is observed. Our largest post-perihelion rate is a factor of ~ 10 larger than that derived for 24 December 1985 (this was near the time of an outburst in cometary activity), and a factor of ~ 25 larger than the value derived for 22 December 1985. In contrast, the water production rates derived from the IUE observations⁹ of Halley near 1 AU suggest that there is virtually no pre- to post-perihelion asymmetry. Because only relative measurements using the same instrument are important in determining the pre- to post-perihelion asymmetry, the discrepancy cannot be attributed to absolute calibration uncertainties. Different sensitivities of the infrared and ultraviolet data to temporal activity may account for the discrepancy, but, failing this, the relationship between OH and H_2O in the cometary coma may require reinterpretation.

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Identification of [Fe III] in the solar ultraviolet spectrum

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Solar ultraviolet spectra have been obtained with high spectral and spatial resolution using the Naval Research Laboratory's high-resolution telescope and spectrograph¹ (HRTS) flown on rockets since 1975 and most recently on the Spacelab 2 Shuttle flight. Because the solar spectrum between $\sim 1,170$ and $1,719 \text{ \AA}$ has been well observed for some years, few lines of substantial intensity remain unidentified. (See for example the recent compilation by Sandlin *et al.*²) The longest exposures during the first rocket flight of the HRTS, which obtained spectra with a spatial resolution of ~ 1 arc s along the slit, of length ~ 1 solar radius, and a spectral resolution of $\sim 0.05 \text{ \AA}$, showed a number of weak emission lines at the solar limb, in particular between $1,570$ and $1,600 \text{ \AA}$ (see Plates 16 and 17 in ref. 3), that could not readily be identified. Improved observations of these lines were obtained during the flight of HRTS on the Spacelab 2 Shuttle flight in July–August 1985, by making longer exposures (60, 100 and 250 s). The scope of the data obtained has been described⁴. We have now identified the emission lines concerned as forbidden (electric quadrupole and magnetic dipole) transitions in Fe III, the first detection of these particular transitions in any source. They must now be considered potential candidates for previously unidentified lines in other low-density ($N_e \leq 10^{10} \text{ cm}^{-3}$) astrophysical sources.

The wavelengths of the emission lines are given in Table 1. Three groups of lines occur, the strongest between $1,575$ and $1,598 \text{ \AA}$, the others between $1,503$ and $1,518 \text{ \AA}$ and between $1,431$ and $1,452 \text{ \AA}$. The wavelengths were determined on a scale established from nearby lines of neutral atoms. For the group around $1,575$ to $1,598 \text{ \AA}$ these were lines of Si I (ref. 5). The wavelength accuracy is estimated as $\pm 0.02 \text{ \AA}$ for lines in this group and $\pm 0.04 \text{ \AA}$ for the weaker lines. Where Sandlin *et al.*² include the lines their wavelengths agree with those listed to within these limits. Several of the lines under discussion are at wavelengths close to those of lines that appear on the disk or in plage and sunspot spectra from the HRTS I flight. However, their behaviour at the limb is quite distinct and unlike the typical appearance of the species, such as neutral atoms, with which they could be blended. The lines are observed only at and above the solar limb and are not detectable more than ~ 20 arc s inside the limb. It was this unusual feature that first attracted attention. In the Spacelab 2 spectra the lines are most clearly observed when the spectrograph slit was set to pass radially through the north solar polar coronal hole. They then extend farther above the limb than in 'quiet' regions. Figure 1 shows one such spectrum (HRTS Spacelab plate 266, 60 s exposure). Although weak, the lines extend farther above the limb than nearby stronger lines of C I and Fe II, indicating a degree of ionization greater than that of Fe II. This conclusion is supported by a series of spectra, also obtained during the Spacelab mission, where the spectrograph slit was set tangential to the limb, centred close

Table 1 Transitions of [Fe III] in solar limb spectra

Observed* λ (Å)	Predicted λ (Å)	Transition $J_{\text{lower}}-J_{\text{upper}}$	Notes
		a^5D-a^5G	
1,575.62	1,575.64	4 5	weak
1,576.68	1,576.66	4 6	strongest line
1,585.99	1,586.03	3 4	blend on disk with Fe II (see ref. 2)
1,586.52	1,586.54	3 5	
1,593.50	{1,593.50 1,593.48}	{2 3 2 2}	
1,593.67	1,593.68	2 4	blend on disk with Fe II and Ni II (see ref. 2)
1,598.42	1,598.41	{1 3 1 2}	blend on disk with Fe II (see ref. 2)
		a^5D-a^5P	
1,503.23	1,503.24	4 2	blend on disk
1,504.53	1,504.56	4 3	strongest in multiplet
1,511.58	1,511.59	3 1	blend in HRTS I spot
1,514.48	1,514.50	3 3	
1,518.51	1,518.54	2 1	v. faint
		a^5D-b^5D	
1,431.93	{1,431.91 1,431.89}	{4 3 4 2}	
1,434.77	1,434.81	4 4	
1,443.84	1,443.84	3 4	blend on disk
1,447.20	{1,447.20 1,447.22}	{2 2 2 3}	blend on disk
1,451.27	1,451.27	1 2	v. faint, blend on disk
1,452.32	1,452.31	1 1	blend on disk

* Accuracy estimated as ± 0.02 Å for lines in a^5D-a^5G , and ± 0.04 Å for other lines.

to the boundary of the north polar coronal hole. The slit was stepped from inside the limb (at -5 arc s) to above the limb (up to $+17$ arc s) in increments of 1 arc s (up to $+5$ arc s) and then 2 arc s. Although the absolute pointing accuracy has yet to be determined, the stigmatic spectra obtained show clearly the reduction in the extent of the chromospheric continuum and successive reduction in the length of lines from different stages of ionization as the slit was moved to greater heights. The Si I recombination continuum ($\lambda \leq 1,520$ Å) and lines of Si I, formed in the solar chromosphere, are restricted to the lowest heights. Higher ionization or excitation lines (for example those of C IV at 1,548 and 1,550 Å and He II at 1,640 Å) extend farthest out into the corona. The lines of Fe II and Si II are intermediate in their behaviour. The lines of [Fe III] can be seen only near the edges of the spectra, indicating strong limb brightening. They extend farther than Si II lines of comparable strength, are broader than the Si I lines and extend at least as far as much stronger lines of Fe II.

The strong limb brightening of the lines shows that they are optically thin transitions, behaviour shared with semi-forbidden electric dipole lines, for example the $^3S-^5S$ lines of O III at 1,661 and 1,666 Å. This indicates transitions either in an abundant species of low oscillator strength or in a species of low abundance. The spatial behaviour suggests at least a second or third stage of ionization. The available spatial information is consistent with the present proposal of lines of [Fe III].

The term scheme of Fe III^{6,7} is illustrated in a partial way in Fig. 2. It is unusual in that states of even parity in the $3d^6$ and $3d^54s$ configurations extend to $80,000$ cm⁻¹ above the ground state before the first term of odd parity occurs. Thus permitted resonance transitions (to the ground a^5D term) do not begin until wavelengths $\leq 1,132$ Å. Permitted transitions at longer

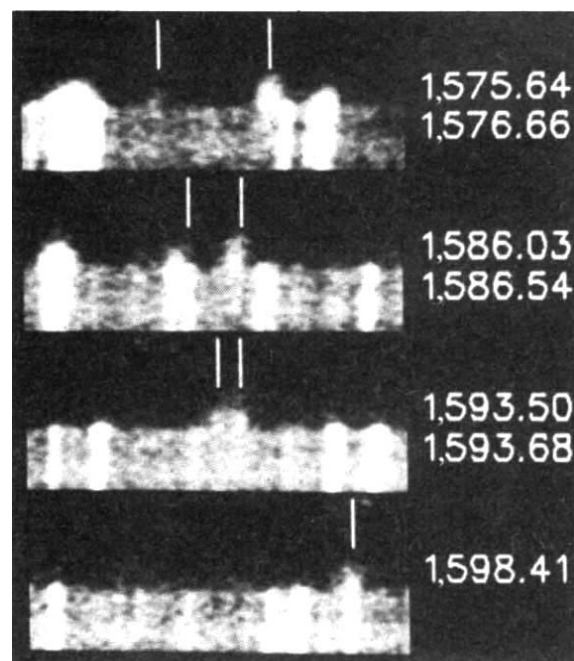


Fig. 1 Sections of a solar limb spectrum obtained with the HRTS instrument on the Spacelab 2 Shuttle flight. HRTS plate 266, a 60 s exposure with the slit radial, crossing the north polar coronal hole. The spectra as illustrated extend about 25 arc s on to the Sun. The wavelengths are given in ångströms. The wavelength increases to the right.

wavelengths occur only to even parity states well above the ground state. The solar lines are transitions within the multiplets $3d^6 a^5D$ to $3d^5 (a^4G) 4s a^5G$, to $3d^5 (a^4P) 4s a^5P$ and to $3d^5 (a^4D) 4s b^5D$ and include both electric quadrupole and magnetic dipole transitions. (Only one other quintet term, $3d^3(^6S)4s ^5S$, lies between a^5G and the ground a^5D term. Transitions between a^5D and a^5S would lie between 2,439 and 2,502 Å, outside the range of the HRTS instrument.) Fe III is a well-known emitter of forbidden lines in the optical spectra of gaseous nebulae and peculiar stars (see for instance refs 8 and 9). However, this is the first identification of [Fe III] in the vacuum ultraviolet region. Table 1 shows the observed and predicted wavelengths. The strongest line is in the a^5D-a^5G multiplet between $J=4$ and $J=6$, as one might expect for a quadrupole transition where $\Delta L=2$ (see ref. 8).

The intensity calibration of the Spacelab 2 spectra is still in progress, but the order of magnitude of the line intensities can be found from previous spectra². The strongest [Fe III] lines have intensities at the limb ~ 15 erg cm⁻² s⁻¹ sr⁻¹, about an order of magnitude weaker than the strongest lines of Fe II and a factor of ~ 100 weaker than the C IV resonance lines.

Until transition probabilities and collision cross-sections are available it is not possible to make definitive statements concerning the excitation of the lines. At present these atomic data have been calculated only for optical forbidden transitions^{8,9} or for electric dipole transitions¹⁰. The [Fe III] lines are likely to be formed at $T_e \approx 20$ –30 kK according to ion-balance calculations.¹¹ The electron density in a coronal hole at this temperature is $N_e \approx 10^{10}$ cm⁻³ (see for instance ref. 12). From the calculations by Garstang *et al.*⁹, collision strengths, Ω , between ~ 1 and 0.3 are typical for transitions with $\Delta L=0, 1$ and 2 and $\Delta J=0, 1$ and 2 . If a mean value of $\Omega=0.5$ is adopted then it can be shown that collisional de-excitation of the upper levels will exceed spontaneous radiative decay unless $A > 40$ s⁻¹. The transitions listed in Table 1 can occur either by electric quadrupole radiation ($\Delta J=2$) or by both magnetic dipole and electric quadrupole radiation ($\Delta J=0, 1$). The largest transition prob-

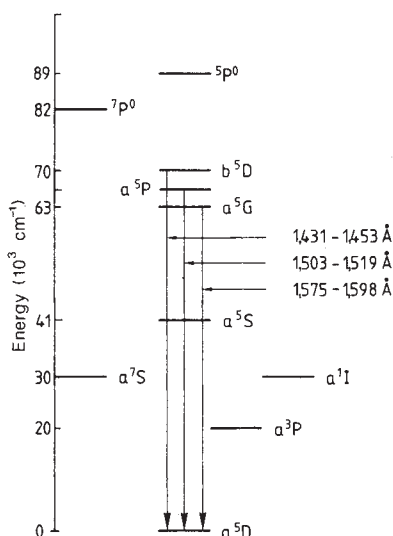


Fig. 2 A partial term scheme for Fe III, showing the observed transitions, some lower-lying terms of the same even parity and the first two terms of odd parity. Term energies are given in cm^{-1} .

abilities found by Garstang⁸ for lower-lying levels are in each case ~ 1 . Thus it is likely that the upper levels have populations close to their Boltzmann values with collisional de-excitation rates exceeding radiative decay rates. Then the ratio of the [Fe III] line fluxes to those of permitted transitions would be sensitive to the electron density. Detailed calculations of the atomic data are required to establish the collisional-radiative regime for the individual lines. The upper levels may attain only a pseudo-Boltzmann population if collisional excitation to higher states of odd parity exceeds the rate for collisional de-excitation to lower levels. In any case the new [Fe III] identifications will provide more information on the structure of the solar chromosphere-corona transition region.

If the a^5G , a^5P and b^5D levels are collisionally de-excited in the solar atmosphere, they could become stronger, relative to permitted transitions of species of similar excitation, in astrophysical sources of lower electron density. For this reason their presence is being investigated in such sources, including the Seyfert galaxy NGC 4151, which has unidentified emission features around 1,575, 1,581 and 1,518 Å (refs 13 and 14). [Fe III] emission is observed in the optical spectrum of NGC 4151^{15,16}. The relative intensities of the quintet transitions in NGC 4151 and other sources cannot be predicted until collision cross-sections and transition probabilities are known, and these are urgently required to establish whether or not the [Fe III] lines are of wider astrophysical significance.

A hierarchical $O(N \log N)$ force-calculation algorithm

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Until recently the gravitational N -body problem has been modelled numerically either by direct integration, in which the computation needed increases as N^2 , or by an iterative potential method in which the number of operations grows as $N \log N$. Here we describe a novel method of directly calculating the force on N bodies that grows only as $N \log N$. The technique uses a tree-structured hierarchical subdivision of space into cubic cells, each of which is recursively divided into eight subcells whenever more than one particle is found to occupy the same cell. This tree is constructed anew at every time step, avoiding ambiguity and tangling. Advantages over potential-solving codes are: accurate local interactions; freedom from geometrical assumptions and restrictions; and applicability to a wide class of systems, including (proto-)planetary, stellar, galactic and cosmological ones. Advantages over previous hierarchical tree-codes include simplicity and the possibility of rigorous analysis of error. Although we concentrate here on stellar dynamical applications, our techniques of efficiently handling a large number of long-range interactions and concentrating computational effort where most needed have potential applications in other areas of astrophysics as well.

Until recently, the dynamics of a system of self-gravitating bodies (the gravitational N -body problem) has been modelled numerically in two fundamentally different ways. The first one, direct N -body integration, involves the computation of all $\frac{1}{2}N(N-1)$ forces between all pairs of particles. This allows an accurate description of the dynamical evolution but at a price that grows rapidly for increasing N . The second way involves a two-step approach: after fitting the global potential field to a special model with a number of free parameters, each particle is propagated in this background field for a short time before the same procedure is reiterated. The potential method involves a number of operations that grow only as $N \log N$. Thus calculations can be performed more quickly, but with a loss of accuracy and generality. The special nature of each potential-solving code is caused by the need to use some technique that is tuned to the geometry of the problem being considered (such as Fourier transforms or spherical or bispherical harmonics²).

Recently, some of the advantages of both approaches have been combined by using direct integrations of force while grouping together increasingly large groups of particles at increasingly large distances. This corresponds to the way humans interact with neighbouring individuals, further villages and increasingly further and larger states and countries—driven by increasing cost and decreasing need to deal with more removed groups on an individual basis. The first implementation of such a hierarchical grouping of interactions was given by Appel³, who used a tree structure to represent an N -body system, with the particles stored in the leaves of the tree. An independent implementation by Jernigan⁴ and Porter⁵ incorporated regularization of close encounters. However, in both codes the logarithmic-growth gain in efficiency comes at the price of introducing additional errors that are hard to analyse because of the arbitrary structure of the tree. Nearby particles may be grouped as leaves of nearby branches, but the phase-space flow of realistic self-gravitating systems demands a continuous updating of the tree structure to avoid tangling and unphysical grouping, requiring complicated book-keeping. It is not at all clear how to understand and estimate the errors caused by the process of approximating lumps of particles together as single pseudo-particles, because individual lumps can take more or less arbitrary shapes and sizes.

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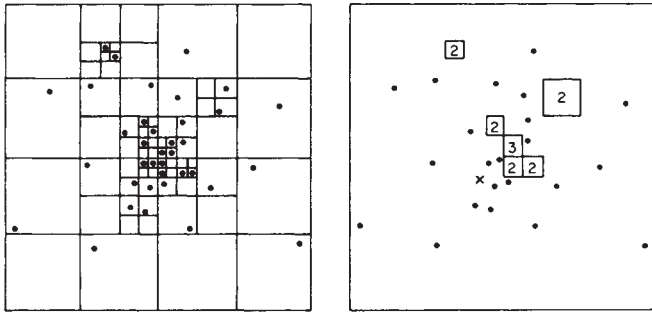


Fig. 1 Hierarchical boxing and force calculation, presented for simplicity in two dimensions. On the left, a system of particles and the recursive subdivision of system space induced by these particles. Our algorithm makes the minimum number of subdivisions necessary to isolate each particle. On the right, how the force on particle *x* is calculated. Fitted cells contain particles that have been lumped together by our 'opening angle' criterion; each such cell represents a single term in the force summation.

We present here a new way of realizing a tree-based force calculation with logarithmic growth of force terms per particle that avoids the tree-tangling complications mentioned above, allows rigorous upper bounds for errors that arise from neglecting internal lump structure, and also offers a well-defined procedure for estimating more typical, average errors. The essential ingredients are (1) a virtual cubical division of empty space in (sub)cells with daughter cells having exactly half the length, breadth and width of their parent; (2) the construction of the actual tree of cells from the virtual one by (i) discarding empty subcells, (ii) accepting subcells with one occupant, and (iii) recursively dividing shared occupancies in sub-subcells; and (3) performing this reconstruction *ab initio* at every time step.

Given this book-keeping structure, the dynamics are implemented by assigning to every non-empty cell, as well as to higher-order cells containing more than one particle, a (pseudo-)particle that contains the total mass in the cell located at the centre-of-mass of all the particles it contains. Any single real particle feels the force of all (pseudo-)particles in the system that represent a cell small enough and far enough to forego the need of further division, thereby screening all its component (pseudo-)particles.

A computer program that implements the hierarchical force calculation is available from us upon request. It contains less than a thousand lines of C code: 150 lines of definitions, 150 lines for tree construction, 100 lines for force calculation and 100 lines for a simple integrator; the remaining lines handle input-output book-keeping.

In what follows we summarize some of the more technical details. The method we use to compute a force in time of $O(\log N)$ is based on a representation of the mass distribution as a hierarchical tree structure, constructed as follows. Begin with an empty cubical cell big enough to contain the system. One by one, load particles into this 'root' cell. If any two particles fall into the same cell, divide that cell into eight cubical subcells (thus the first such division occurs as soon as the second particle has been loaded in, splitting the system into at least eight pieces). Each divided cell is represented by a data structure that holds information about the subcells it contains: a summary of global physical quantities (mass and centre-of-mass position) as well as pointers to the daughter cells, which may be referenced to obtain more detailed information. Continue this process of subdividing to as high a level as required. When all N particles have been loaded, the system space will have been partitioned up into a number of cubical cells of different sizes, with at most one particle per cell. These particle-bearing cells are grouped together into larger cubical cells, which are grouped together into still larger parent cells, and so on down to the root cell, which contains the entire system. The average size of a particle-

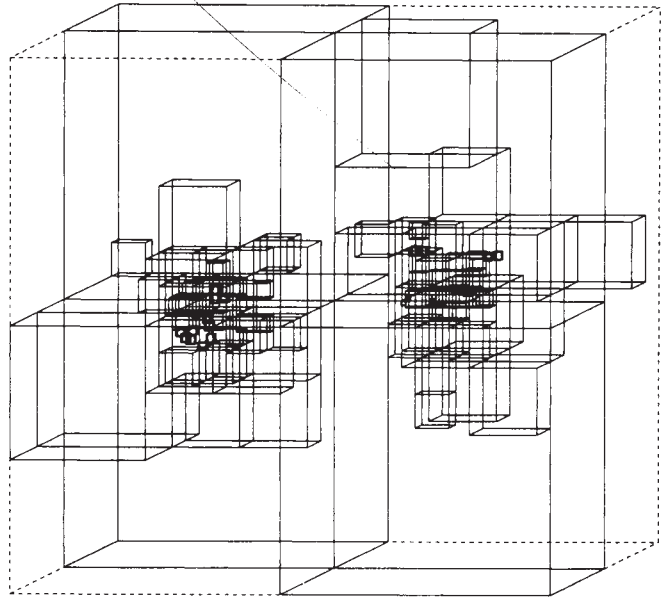


Fig. 2 Box structure induced by a three-dimensional particle distribution. This example was taken from the early stages of an encounter of two $N=64$ systems, and shows how the boxing algorithm can accommodate systems with arbitrarily complicated geometry. The particle distribution corresponding to a system with 32 times as many members is shown in Fig. 3.

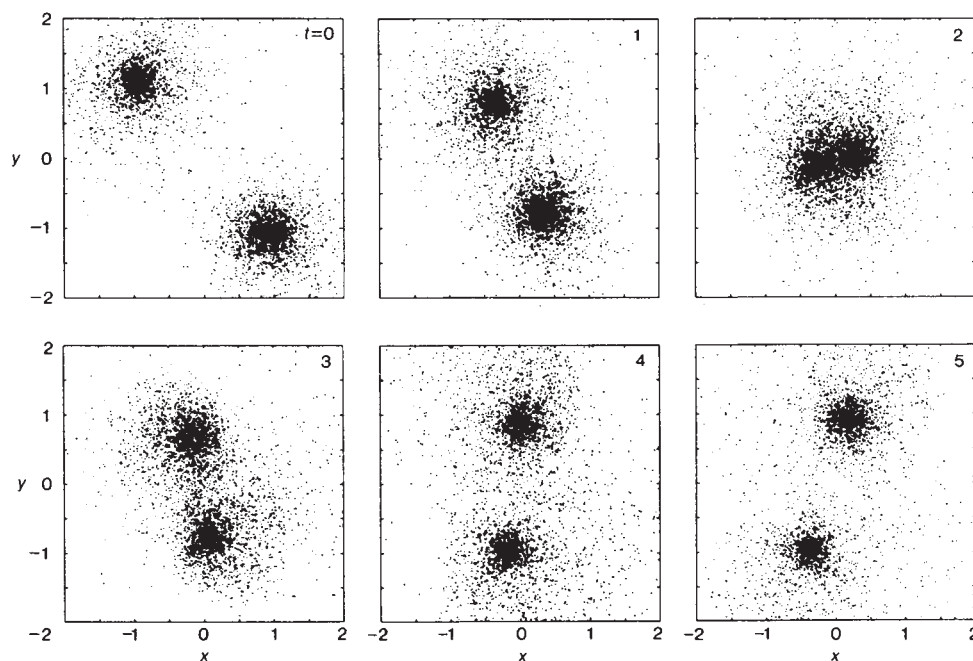
bearing cell is of the order of the interparticle spacing, so the 'height of the tree' (that is, the number of subdivisions required to reach a typical cell, starting at the root), is of $O(\log_2 N^{1/3}) = O(\log N)$, and the time required to construct the tree is of $O(N \log N)$. The final step in constructing the tree is to tag the subdivided cells with the total mass and centre-of-mass position of the particles they contain; by propagating information down the tree from the particles towards the root, this step may also be accomplished in a time of $O(N \log N)$.

Having constructed such a tree, the force on any particle p may be approximated by a simple recursive calculation. Start at the root cell of the tree, which contains the entire system. Let l be the length of the cell currently being processed and D the distance from the cell's centre-of-mass to p . If $l/D < \theta$, where θ is a fixed accuracy parameter ~ 1 , then include the interaction between this cell and p in the total being accumulated. Otherwise, resolve the current cell into its eight subcells, and recursively examine each one in turn. The core of the force calculation routine may be compactly expressed in SCHEME, a dialect of LISP:

```
(define (acceleration particle ensemble)
  (cond ((singleton? ensemble)
        (newton-acceleration particle (the-element ensemble)))
        ((< (/ (diameter ensemble)
              (distance particle (centroid ensemble)))
             theta)
         (newton-acceleration particle (centroid ensemble)))
        (else
         (reduce sum-vector
                  (map (lambda (e) (acceleration particle e))
                       (subdivisions ensemble))))))
```

Note that in LISP, a function with arguments $f(x, y, \dots)$ is written as $(f x y \dots)$. For example, $(\text{newton-acceleration } p_1 \ p_2)$ calls a function to compute the acceleration of particle p_1 due to p_2 . The $(\text{cond } \dots)$ form is a three-way conditional, computing the acceleration directly in the first two cases, and by recursion in the final $(\text{else } \dots)$ clause. Elements of the SCHEME programming language are presented in Abelson *et al.*⁶ Figure 1

Fig. 3 Encounter of two spherical systems, simulated by using our hierarchical acceleration technique in combination with a simple leap-frog integrator. The incoming systems were launched on parabolic orbits, but become bound because of dynamical friction. Note the striking wakes lagging behind the density centres of the two systems at $t=3$, 4 (in our units the gravitational constant, the mass of each galaxy and the total binding energy of the whole system all equal unity). With a total of $N=4,096$ particles in the system and an opening angle criterion of $\theta=1$, the number of two-body interactions computed by our technique is less than 0.1 of the $\frac{1}{2}N(N-1)$ required by a direct-summation force calculation. The calculation took 10 h on a VAX 11/780 (in double precision because of compiler limitations; a single-precision calculation would take half as long). With a time step $\Delta t=0.05$ and softening parameter $\epsilon=0.025$, energy was conserved to $\sim 1\%$.



illustrates this process for a small number of particles in two dimensions; increasing the number to $10^4 \sim 10^5$ in three dimensions typically increases the number of interactions per particle to only of order 10^2 .

The number of interactions considered by this procedure in computing the force on p is of order $\log N$ for large N . Suppose the mass distribution is homogeneous within the root cell. Increasing the total number of particles eightfold is roughly equivalent to adjoining eight similar root cells together. The seven new cells not containing p will contribute some 'relatively small' number ΔN_i of additional terms to the force approximation. Now the expectation value $\langle \Delta N_i \rangle$ depends on θ , but not on the total number of particles or the size of the system. Thus the time required to calculate the force on a particle increases by a constant increment (of $\langle \Delta N_i \rangle$), whereas N increases by a constant factor (of eight). In other words, the time required by the CPU (central processing unit) to compute the force on a single particle is on the scale of $O(\log N)$.

A rigorous error analysis of the force-calculation algorithm is possible because our prescription yields a unique, well-characterized tree structure based on up-to-date particle positions. Each compound cell that we choose not to subdivide introduces a small error due to quadrupole and higher-order moments of the mass distribution within the cell (the dipole term vanishes when expanding around the centroid). The magnitude of this error may be bounded by a 'worst-case' analysis for which the quadrupole moment is maximized (for example, two lumps placed in opposite corners of the cell), and estimated from an analysis of root-mean-square fluctuations within each cell together with estimates of the coherence time scales for these fluctuations. We shall present this analysis in a more detailed paper. In practice, forces computed even with an opening angle parameter as large as $\theta=1$ are still accurate to $\sim 1\%$ with little dependence on N . Empirically, we find the force error scales approximately as the -1.5 power of the computing time. These errors are only weakly correlated from one time step to the next, resulting in a build-up close to a random walk rather than a steady drift.

As a test of our new method, we have written a simple N -body code using our force-calculation scheme with a time-centred leap-frog integrator, in which positions and velocities are alternately advanced. A parabolic encounter of two galaxies is initi-

ated at a distance of several galactic radii, leading to a box structure as shown in Fig. 2. The results of a 4096-body calculation of such an encounter are shown in Fig. 3. This calculation took 10 h of CPU time on a VAX 11/780 with a floating point accelerator.

There are several ways in which the code can be made more efficient. We are now investigating these, and we shall discuss our results in detail elsewhere. We just mention three possible improvements: (1) using a higher-order integration scheme such as Aarseth's fourth-order polynomial method rather than our second-order leap-frog method, which will require careful adjustments to avoid glitches caused by discrete differences between the grouping of particles in cells from one time step to the next (for example by multiply covering space in partly overlapping virtual grids); (2) including quadrupole moments in the description of cells as pseudoparticles characterized by the total mass in the cell as located in the centre of mass; (3) introducing individual time steps for particles which undergo strongly changing interactions, which could be accomplished by subsequently halving the time step when needed—thus extending the three-dimensional spatial halving of cells to a four-dimensional space-time division in rectangular subcells.

An interesting aspect of our new code is the different emphasis it places both on software and hardware, in comparison with other codes. On the hardware side, the hierarchical structure of our code does not lend itself easily to vectorization (although this may well be worth exploring). In contrast, we expect our code to be most useful on computers with highly parallel architectures (with one processor per particle, computer time is reduced approximately by a factor of N). On the software side, the hierarchical decomposition of the problem is best realized by using recursive descriptions. Recursive function calls and other general control and data structures are not well supported or clearly represented in FORTRAN. This has led us to consider other programming languages such as C, PASCAL and LISP. Another advantage offered by these languages is that they permit a clarity of presentation of our ideas, which makes the underlying techniques available to other researchers. Of course, if a particular computer has a FORTRAN compiler which is an order of magnitude faster than other compilers, it makes sense to translate a version of our program into FORTRAN, trading clarity and modularity for efficiency.

Our application to *N*-body calculations is only one in a range of possibilities including the calculation of radiation fields (replacing particles with sources) and self-gravitating fluid flow (cell division being governed by the complexity of the local flow pattern). Thus our technique forms a general tool for simultaneously handling a large number of long-range interactions and for concentrating computing resources locally where most needed.

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The 400-km seismic discontinuity and the proportion of olivine in the Earth's upper mantle

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The 400-km seismic discontinuity has traditionally been ascribed to the isochemical transformation of α -olivine to the β -modified-spinel structure in a mantle of peridotitic bulk composition^{1–6}. It has recently been proposed^{7,8} that the observed seismic velocity increase at 400 km depth is too abrupt and too small to result from a phase change in olivine but instead requires that the transition zone be chemically distinct in bulk composition from the uppermost mantle. By requiring phase relations in the Mg_2SiO_4 - Fe_2SiO_4 system to be internally consistent thermodynamically, we find that the α - β transition in olivine of mantle ($\text{Mg}_{0.9}\text{Fe}_{0.1}$) $_2\text{SiO}_4$ composition is extremely sharp, occurring over a depth interval (isothermal) of ~ 6 km. The magnitude of the predicted velocity increase is in agreement with that observed seismically^{9,10} if the transition zone is composed of ~ 60 –70% olivine. Thus, our results indicate that seismic velocities across the 400-km discontinuity are consistent with a transition zone of homogeneous peridotitic composition and do not require chemical stratification.

The 400-km seismic discontinuity reflects a change in elastic properties of the mantle and has been attributed to a phase transformation of olivine to a spinel-like structure at high pressures¹. Subsequent work has given rise to a generally accepted model in which the discontinuity is attributed to such an isochemical phase change in a mantle of homogeneous olivine-rich, or peridotitic, composition^{2–6}. This model has the advantage of simplicity and can be tested experimentally.

Recently, it has been suggested^{7,8} that a phase transition in olivine would produce a gradual velocity increase over an appreciable depth interval—rather than the abrupt increase observed seismically—and that the magnitude of the increase would be more than twice that actually observed. It was proposed that the seismic data require the transition zone to be chemically distinct in bulk composition from the uppermost mantle, with the transition zone consisting of a pyroxene-garnet rich 'piclogite' composition containing either 16%⁷ or 30%⁸ olivine. The 400-km discontinuity is ascribed to either a change

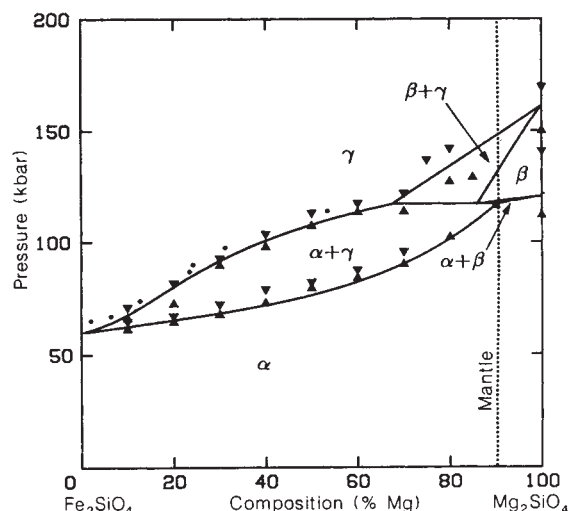


Fig. 1 Isothermal pressure-composition diagram showing calculated boundaries for olivine polymorph stability fields at 1,273 K. Also shown are experimental data points^{12–14,18–20} delimiting the high-pressure stability limits of the low-pressure assemblages (▲), the low-pressure stability limits of the high-pressure assemblages (▼), and the compositions of γ phase which coexist with α phase at the indicated pressures (●). Dashed line shows ($\text{Mg}_{0.9}\text{Fe}_{0.1}$) $_2\text{SiO}_4$ composition.

in chemical composition from peridotite to underlying piclogite or—if this peridotite/piclogite boundary is referred to shallower depths—to the transformation of pyroxene to a garnet-like structure.

In a previous study¹¹, we showed that the transformation of pyroxene to a garnet structure would produce a smooth and gradual increase in seismic velocity, rather than the discontinuity observed at 400 km. In the present study, we have examined the olivine-spinel phase transitions to determine whether the observed seismic velocity variations may be attributable to such a phase change. The α -olivine to β -modified-spinel transition has been commonly represented by a broad ' $\alpha + \beta$ divariant loop', a region in which both phases coexist in stable equilibrium. If this representation were accurate, the α phase would transform to the β phase in a continuous and gradual manner, and this phase change would not produce a sharp discontinuity in seismic velocity. However, the available experimental data (Fig. 1) do not constrain the width of this $\alpha + \beta$ loop, since no high-pressure experiments have yet produced both phases together in equilibrium for olivine of mantle ($\text{Mg}_{0.9}\text{Fe}_{0.1}$) $_2\text{SiO}_4$ composition. We have used available thermoelastic and calorimetric data on the olivine polymorphs (α -olivine, β -modified-spinel, and γ -spinel) to constrain the width of the $\alpha + \beta$ divariant loop. By requiring the phase diagram for the Mg_2SiO_4 - Fe_2SiO_4 system to be internally consistent thermodynamically, we have attempted to determine the sharpness and magnitude of a seismic discontinuity resulting from a phase change in olivine.

If the partial molar free energies of Mg_2SiO_4 and Fe_2SiO_4 components are known as functions of pressure, temperature, and composition, then the boundaries of the stability fields for the various phase assemblages (α , $\alpha + \beta$, β , $\beta + \gamma$ and so on) can be calculated explicitly. To compute the free-energy functions, we require knowledge of the enthalpies, entropies, volumes, and solution activities of the components in the various phases at the pressures, temperatures and compositions of interest. We used the available experimentally-measured values of the enthalpies and entropies^{5,12–14}, heat capacities¹⁵, molar volumes and coefficients of thermal expansion⁴, elastic moduli¹⁶, and activity coefficients¹⁷ for the phases and components in question. Where measured values were extremely uncertain or

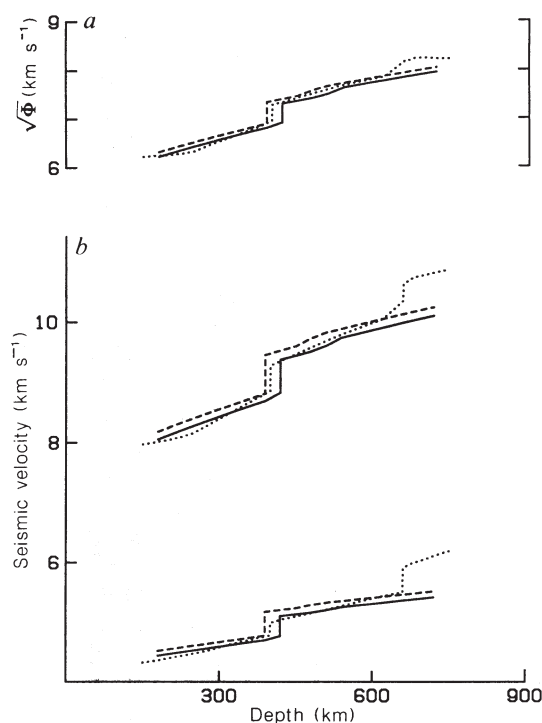


Fig. 2 *a*, Calculated bulk sound velocity ($\sqrt{\Phi}$) as a function of depth for a mantle consisting of pure olivine of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ composition, along 1,700 K (dashed line) and 2,000 K (solid line) isotherms. Composite seismic profile GCA + TNA^{9,10} (dotted line) is shown for comparison. *b*, Calculated P- and S-wave velocities (V_p and V_s , respectively) as functions of depth for a mantle consisting of pure olivine of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ composition, along 1,700 K (dashed line) and 2,000 K (solid line) isotherms. Composite seismic profile GCA + TNA (dotted) is shown for comparison.

unavailable (such as for the metastable β - Fe_2SiO_4 component), we estimated them by formally inverting the calculation procedure and requiring the estimated values to reproduce the observed phase relations as constrained by the available synthesis data^{12-14,18-20}. (Detailed discussions of the experimental data, the methods of computation and the requirements of thermodynamic consistency are presented elsewhere²¹.) Using the Grüneisen thermal state equations²² and the third-order Birch-Murnaghan isothermal state equation²³ to compute the temperature and pressure dependencies, respectively, of the molar volumes, we computed the boundaries of the stability fields in pressure-composition space for the various assemblages along the Mg_2SiO_4 - Fe_2SiO_4 join, as shown in Fig. 1.

The resulting phase diagram is internally consistent thermodynamically and is consistent with the available calorimetric, thermoelastic and synthesis data, within their experimental uncertainties. Note that the $\alpha + \beta$ divariant loop is extremely narrow, ~ 2 kbar wide, so that the $\alpha \rightarrow \beta$ transition in mantle olivine would be quite sharp, occurring over a depth interval of ~ 6 km. The width of this transition is extremely robust to uncertainties in the data set; extreme perturbations of the various parameters yield a variation in transition width of only ± 1 kbar (~ 3 km).

Using the method of free-energy minimization²⁴, we have calculated stable phase assemblages and associated (Voigt-Reuss-Hill averaged²⁵) bulk sound velocities ($\sqrt{\Phi}$) as functions of pressure (depth) along 1,700 K and 2,000 K isotherms for a model mantle consisting of pure olivine of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ composition (Fig. 2a). The model bulk sound velocities for a pure olivine mantle agree quite well (within $\pm 0.06 \text{ km s}^{-1}$) with

those calculated from the representative high-resolution P-wave and S-wave velocity profiles GCA⁹ (Gulf of California) and TNA¹⁰ (Tectonic North America), respectively. (As we are not attempting to model the 670-km discontinuity, we have not included the effects of any higher pressure phases, such as the silicate perovskite, which might contribute to velocity increases at that depth².) A sharp jump in velocity occurs at ~ 400 km depth due to the $\alpha \rightarrow \beta$ transition. A small kink in the velocity profile occurs at ~ 550 km due to the $\beta \rightarrow \gamma$ transition, but this slight velocity change is barely detectable due to the similarities of the elastic properties of the β and γ phases¹⁶. The ratio of the magnitude of the observed (GCA + TNA) velocity increase at 400 km (4.9%) to that calculated for the model pure olivine mantle (6.6%) suggests that the mantle in the transition zone contains $\sim 74\%$ olivine.

We have separated our values of bulk sound velocity ($\sqrt{\Phi}$) into P- and S-wave velocities for a model pure olivine $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ mantle (Fig. 2b). This requires additional assumptions because the temperature and pressure derivatives of the shear moduli of the high-pressure polymorphs are unknown²⁶. To perform the decomposition, we first computed velocities by assuming that the behaviour of the shear moduli at high pressures and temperatures follows the same pattern as that of the bulk moduli, namely that the pressure derivatives and the logarithmic temperature derivatives, respectively, are the same²² for all the phases of a given component. In a second approach, we followed previous workers²⁷ in extracting P- and S-wave velocities by assuming that Poisson's ratio for our model pure olivine mantle was the same, at corresponding depths, as that observed (GCA + TNA) seismically. These independent approaches yielded virtually identical results and provide support for our conclusions. The magnitudes of the observed (GCA + TNA) velocity increases at 400 km (4.8 and 4.5% in P- and S-wave velocity, respectively), and those calculated for the model pure olivine mantle (7.2 and 8.1%), are indicative of a mantle composed of $\sim 62\%$ olivine (a peridotitic mantle), in excellent agreement with the results of Weidner⁶.

Thus, an isochemical phase change from α -olivine to β -modified-spinel in olivine of mantle composition would produce a sharp 400-km seismic discontinuity ~ 6 km wide. In fact, our computed velocity increase is even sharper than that required by current seismic observations. The actual sharpness of this discontinuity is difficult to resolve seismically^{28,29}. Leven³⁰ suggests that the discontinuity occurs over a depth interval of ~ 6 km; other studies³¹ suggest that the discontinuity is at least 10 km wide. Our transition width of $6(\pm 3)$ km is computed for isothermal conditions; temperature gradients through the transition could broaden the discontinuity⁴ by up to ~ 7 km.

Thus, seismic velocities above and below a 400 km depth for a model mantle consisting of pure olivine of $(\text{Mg}_{0.9}\text{Fe}_{0.1})_2\text{SiO}_4$ composition are in good agreement with those observed seismically^{9,10}. The magnitudes of the abrupt increases in bulk sound velocity and in P- and S-wave velocities at 400 km are consistent with a mantle composed of between 62 and 74% olivine. Since phase transformations from pyroxene to garnet would not produce such a discontinuity¹¹, a mantle dominated compositionally by olivine appears necessary for the generation of a sharp 400-km discontinuity unless arbitrary changes in bulk chemical composition are invoked. We therefore conclude that seismic observations are consistent with a peridotitic mantle to a depth of at least 650 km and thus do not require the transition region to be chemically distinct from the overlying mantle.

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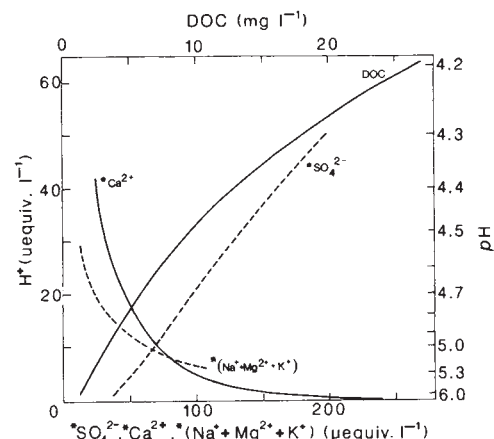


Fig. 1 Partial regressions of H^+ on DOC, $*SO_4^{2-}$, $*Ca^{2+}$ and $*(Na^+ + Mg^{2+} + K^+)$.

Natural and anthropogenic causes of lake acidification in Nova Scotia

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Controversy has arisen over the recent acidification of lakes^{1–4}, ascribed by many to anthropogenic acid deposition from the atmosphere^{5,6}, and by some to natural processes of soil acidification enhanced by the regrowth of forests after cutting and burning^{7–10}. Here we show, by analysing the chemistry of Nova Scotian lakes and ponds on base-poor terrains, that both anthropogenic and natural acidification can be important. We calculated correlations and regressions between hydrogen ion (H^+) concentrations and each of four predictors: dissolved organic carbon (DOC) (a surrogate for complex coloured organic acids, often of high molecular weight^{11,12}), non-marine sulphate (denoted by the prefixed asterisk as $*SO_4^{2-}$; a surrogate for acid deposition¹³), non-marine calcium ($*Ca^{2+}$; the major basic cation from soil weathering and ion exchange), and the sum of the other non-marine base cations sodium, magnesium and potassium ($*(Na^+ + Mg^{2+} + K^+)$). The results indicate that acidity in these waters is affected both by organic acids from peatland catchments and by acid deposition from long-range and local sources (see ref. 14).

Previous investigators ascribed acidity of lakes on diverse geological substrates in Halifax County to either peaty inflows¹⁵ or acid deposition¹⁶. Both used methods inadequate for measuring SO_4^{2-} in coloured waters¹⁷, so another study was needed. We sampled lakes on granite and quartzite terrains between 2.5

and 42 km around the Halifax–Dartmouth power plant, whose annual emissions declined from 21.5 kton SO_2 in 1980 to 8.3 kton in 1984 (ref. 18). Thirty-seven surface waters were collected during 7–18 December 1984 after a dry period (September–November precipitation, 42% normal) and filtered through Reeve–Angel no. 934AH glass-fibre filters before analysis. H^+ was measured by a Radiometer combination electrode, DOC by photo-oxidation, gas dialysis and colorimetric titration with phenolphthalein, and Ca^{2+} , Mg^{2+} , Na^+ and K^+ by atomic absorption spectrophotometry. SO_4^{2-} was analysed by ion chromatography and colorimetrically by methyl thymol blue after photo-oxidation; results were averaged. (Ion chromatography yielded a mean 1.2% above that of colorimetry, r^2 was 0.97, the slope of the relation departed by only 2.5% from the 1:1 line, and the average difference between techniques was 6 $\mu\text{equiv. l}^{-1}$.) $*SO_4^{2-}$, $*Ca^{2+}$ and $*(Na^+ + Mg^{2+} + K^+)$ were estimated as differences between total and marine concentrations, the latter from ratios to chloride (Cl^-) in sea water. Cl^- was measured by ion chromatography and colorimetrically by mercuric thiocyanate; results were averaged. (Colorimetry yielded a mean 1.4% above that of ion chromatography, r^2 was 0.99, but the slope of the relation departed from the 1:1 line by 8%, so that at very low concentrations colorimetric values were 15% below those of ion chromatography, whereas at very high concentrations colorimetric values exceeded those of ion chromatography by almost 5%. Average difference between techniques was 17 $\mu\text{equiv. l}^{-1}$.) Colour was measured as absorbance at 320 nm in a 1-cm cell ($A_{320}^{1\text{cm}}$), and in Pt/Co units. Because of plant uptake and microbial reduction, nitrate and ammonium ions—important components of acid deposition—were negligible (N usually $<0.01 \text{ mg l}^{-1}$). Alkalinity was usually <2 and always $<25 \mu\text{equiv. l}^{-1}$. Ranges of chemical properties were: DOC, 0.7–220 mg l^{-1} ; colour ($A_{320}^{1\text{cm}}$), 0.001–0.574 and (Pt/Co units), 7–200; H^+ , 1–98 $\mu\text{equiv. l}^{-1}$; Cl^- , 90–832 $\mu\text{equiv. l}^{-1}$; $*SO_4^{2-}$, 12–203 $\mu\text{equiv. l}^{-1}$; $*Ca^{2+}$, 17–239 $\mu\text{equiv. l}^{-1}$; and $*(Na^+ + Mg^{2+} + K^+)$, 2.4–105 $\mu\text{equiv. l}^{-1}$. In the last case, mean percentages were: $*Na^+$, 64; $*Mg^{2+}$, 23; $*K^+$, 13.

We analysed the chemistry of 33 waters statistically, excluding four lakes by standard diagnostic criteria for regressions¹⁹. Data were transformed to linearize relationships between H^+ and the predictors; error distributions were also normalized. H^+ was transformed to its square root, other data to natural logarithms. Transformed means were: H^+ , 3.93; DOC, 1.50; $*SO_4^{2-}$, 4.43; $*Ca^{2+}$, 4.03; and $*(Na^+ + Mg^{2+} + K^+)$, 3.60.

In the transformed scales, DOC is the best of the four predictors of H^+ : its correlation coefficient is 0.746 and its partial correlation coefficient—in an equation including $*SO_4^{2-}$, $*Ca^{2+}$ and $*(Na^+ + Mg^{2+} + K^+)$ —is 0.908. (Except where specified, all correlation coefficients are significant at $p < 0.01$.) For $*SO_4^{2-}$ the corresponding statistics are lower, with a correlation

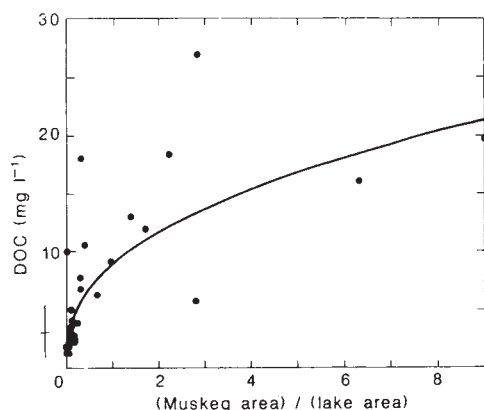


Fig. 2 Relation between DOC and the ratio (muskeg area)/(lake area) ($n=30$). Cross at lower left indicates mean and range of sites without muskeg. Logarithmic regression, $\ln \text{DOC} = 0.395 \ln \{(\text{muskeg area})/(\text{lake area})\} + 2.19$; correlation, $r_{\ln}^2 = 0.605$.

coefficient of 0.095 (not significant) and a partial correlation coefficient of 0.742. $^*\text{Ca}^{2+}$ predicts H^+ better than $^*\text{SO}_4^{2-}$, with correlation coefficients of -0.385 ($p < 0.05$) and -0.849 respectively, negative because of alkalinity associated with $^*\text{Ca}^{2+}$ released from soils and sediments. Coefficients for $^*(\text{Na}^+ + \text{Mg}^{2+} + \text{K}^+)$ shift sign after partial correlation, from 0.386 ($p < 0.05$) to -0.558 . The multiple regression equation, including both natural and anthropogenic acidifying agents and neutralizing base cations as predictors, is

$$\sqrt{\text{H}^+} = 1.33 + 2.26 \ln \text{DOC} + 3.66 \ln ^*\text{SO}_4^{2-} - 2.97 \ln ^*\text{Ca}^{2+} - 1.40 \ln ^*(\text{Na}^+ + \text{Mg}^{2+} + \text{K}^+) \quad (1)$$

(DOC in mg l^{-1} , other variables in $\mu\text{equiv. l}^{-1}$). The multiple correlation coefficient ($R^2 = 0.878$) accounts for most of the variance in H^+ , and is distinctly greater than that ($R^2 = 0.823$) for H^+ on DOC, $^*\text{SO}_4^{2-}$ and $^*\text{Ca}^{2+}$.

Another possible cause of lake acidification is the deposition of sea salts, whose metal cations can displace adsorbed H^+ episodically and seasonally from soils and peats into streams and lakes²⁰. Cl^- can be used as a predictor of the sea-salt effect on H^+ , and when substituted for $^*(\text{Na}^+ + \text{Mg}^{2+} + \text{K}^+)$ in equation (1), it too yielded a correlation ($R^2 = 0.857$) distinctly greater than that with three predictors. However, when Cl^- was added to equation (1) as a fifth predictor, the correlation ($R^2 = 0.885$) did not improve appreciably.

Figure 1 illustrates partial relations of H^+ to DOC, $^*\text{SO}_4^{2-}$, $^*\text{Ca}^{2+}$ and $^*(\text{Na}^+ + \text{Mg}^{2+} + \text{K}^+)$ over their observed ranges of concentration, holding the other three predictors at their mean values in equation (1). DOC has a broader range of effect than $^*\text{SO}_4^{2-}$ on H^+ , which rises from 1 to $64 \mu\text{equiv. l}^{-1}$ over the full range of DOC increase and from 1 to $50 \mu\text{equiv. l}^{-1}$ over the full range of $^*\text{SO}_4^{2-}$ increase. $^*\text{Ca}^{2+}$ has a negative effect upon H^+ , which declines from 42 to $<1 \mu\text{equiv. l}^{-1}$ over the full range of $^*\text{Ca}^{2+}$ increase. The decline is curvilinear, reflecting the rapid drop in pH as bicarbonate alkalinity (associated primarily with $^*\text{Ca}^{2+}$) is exhausted. A smaller decrease in H^+ , from 29 to $6 \mu\text{equiv. l}^{-1}$, occurs with increasing $^*(\text{Na}^+ + \text{Mg}^{2+} + \text{K}^+)$.

Correlations among DOC, colour and absorbance are highly significant, indicating humic substances, chiefly fulvic acids^{11,12}, produced by decomposition of plant remains in the catchments. DOC also correlates with the ratio (catchment area)/(lake area), because larger catchments generally contain larger areas of the peatlands that are the major sources of DOC input (see below) and the area of the lake is a major determinant of the volume in which the input is dissolved. The correlation between DOC and the ratio is logarithmic and highly significant ($r_{\ln}^2 = 0.392$). The prediction equation is $\ln \text{DOC} = (-0.095) + 0.634 \ln \{(\text{catchment area})/(\text{lake area})\}$. Catchments have been mapped (by the Nova Scotia Department of Lands and Forests) as muskeg, rock

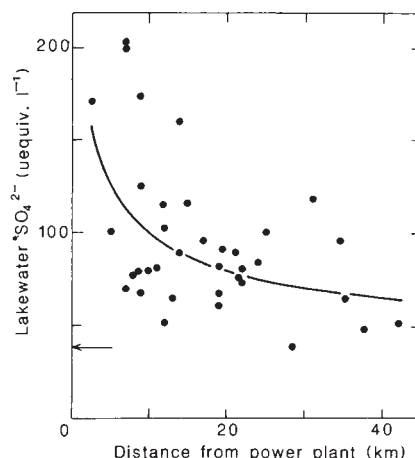


Fig. 3 Decline of lakewater $^*\text{SO}_4^{2-}$ outward from the Halifax-Dartmouth power plant ($n=36$, rejecting one outlier). $\ln ^*\text{SO}_4^{2-} = 5.35 - 0.321 \ln \text{km}$; $r_{\ln}^2 = 0.266$. Horizontal arrow, background from long-distance transport.

barren, softwood ($>75\%$) forest, hardwood ($>75\%$) forest and mixed forest. Muskegs (which we remapped from air-photos, adjusting other areas appropriately) represent open peatland, sometimes with stunted trees, and are commonly dominated by *Sphagnum* moss. Rock barrens include large patches of ericaceous shrubs over acid mor humus on rock or shallow sandy soil, and small patches of *Sphagnum*. Muskegs and softwood forests on peat are the most likely sources of DOC to lakes^{21,22}. Rock barrens are also possible sources because they often have patches of peat, and also lack deeper, less acid B soil horizons capable of precipitating humic substances with oxides of iron and aluminium²³.

For the 16 waters with all three cover types, analysis indicated significant correlation of DOC only with the ratio (muskeg area)/(lake area). For all 30 catchments with muskeg (Fig. 2), the logarithmic correlation and regression of these two variables are highly significant, the correlation accounting for 60% of the variance in DOC. Restriction to lakeside ($n=14$) and lakeside plus streamside muskeg ($n=26$) does not improve the correlation. The arithmetic scatter is, however, substantial, and softwood (black spruce and tamarack) swamps, which could not be mapped separately from upland softwood forests, deserve investigation in this context. The rate of water-flow through peatlands also influences DOC concentrations²¹.

The $^*\text{SO}_4^{2-}$ in these lake waters comes chiefly from acid deposition. (Maps of soil and bedrock geology were inspected carefully to exclude the Halifax slate formation, in which local occurrences of pyrite are a source of $^*\text{SO}_4^{2-}$. However, there is a remote possibility that some $^*\text{SO}_4^{2-}$ is supplied through the use of crushed Halifax slate on gravel roads beyond the extent of its geological exposure²⁴). Figure 3 shows the influence of Halifax-Dartmouth (population 191,000); an oil-fired generating station at Tufts Cove (360 MW) is the reference point for distance. The correlation between $^*\text{SO}_4^{2-}$ and distance is relatively low. Differences in vegetation cover, fluctuations in precipitation, wind speed and direction, and differences in water retention time and reduction of SO_4^{2-} in anoxic bottom waters also influence $^*\text{SO}_4^{2-}$ inputs and concentrations. The curve in Fig. 3 approaches a baseline ($39 \mu\text{equiv. l}^{-1}$) representing long-distance transport of air pollutants; it was calculated by interpolating Halifax along a line connecting average $^*\text{SO}_4^{2-}$ concentrations in lakes at Kejimikujik National Park ($46 \mu\text{equiv. l}^{-1}$) and Cape Breton Highlands National Park ($20 \mu\text{equiv. l}^{-1}$), respectively 140 km SW and 350 km NE of Halifax²⁵.

Interaction of acid deposition with soil organic matter is claimed to decrease the solubility of humic compounds⁸. Halifax County, with a broad range of lake DOC and moderate levels of acid deposition, shows no evidence of this, the correlation

between DOC and $^*SO_4^{2-}$ ($r_{in}^2 = 0.03$) being insignificant (see ref. 26).

Coloured organic acids from catchments strongly influence the acidity of lakes on base-poor substrates, so that even small amounts of humic DOC affect lakewater pH greatly; between minimum DOC at $<1 \text{ mg l}^{-1}$ and the log-normal mean at 4.5 mg l^{-1} , pH declines more than one unit to 4.8 (Fig. 1). This conclusion probably applies throughout Nova Scotia; 769 lakes, mostly on base-poor substrates, exhibit a log-normal distribution of colour (J.K.U. unpublished observations) greater than that of Halifax County lakes (antilog mean = 35 Pt/Co units in Nova Scotia, 27 units in Halifax County). Moreover, in 234 Nova Scotian lakes²⁷, organic anions average $49 \mu\text{equiv. l}^{-1}$ and $^*SO_4^{2-}$ $60 \mu\text{equiv. l}^{-1}$. The influence of coloured organic acids is likely in other areas (for example much of the Precambrian Canadian Shield) on comparably base-poor substrates with an abundance of acid peatlands, and with similar concentrations of organic anions (see ref. 28). Their significance for lake-acidification models^{6,29} is not known and should be evaluated.

Despite the influence of coloured organic acids from natural sources (see refs 14, 26 and 30), the effect of acid deposition on surface waters in Nova Scotia is clear (Fig. 3) and especially important at snow-melt and other periods of high flow^{14,26}. It has probably caused naturally acidified waters to acidify further and beyond thresholds for losses of diatoms³¹ and salmon^{32,33}. The degree of natural acidification, as well as the amount of wet $^*SO_4^{2-}$ deposition from the atmosphere, should be taken into account in determining target loadings for acid deposition.

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A 40,000 year-old human occupation site at Huon Peninsula, Papua New Guinea

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The geographical position of the island of New Guinea suggests that it may have been an early staging post in the Pleistocene settlement of Australia from the Indonesia–Indochina region. Previous data have not supported this, as archaeological sites 35,000 to 40,000 years old occur in southern Australia^{1,2}, whereas the earliest previously known in Papua New Guinea is 26,000 years old^{3–5}. We now report evidence that the north coast of Papua New Guinea was occupied at least 40,000 years ago. Sahuland, which is the greater land area of Australia and New Guinea plus their connecting continental shelf exposed as land when Pleistocene sea levels were lower than now^{6,7}, was occupied by humans in several widely separated areas at that time. A distinctive ‘waisted axe’ culture appears to have existed in New Guinea and probably in Australia in the Late Pleistocene, but antecedents are not yet known from east and southeast Asia. There is evidence for hafting of these tools at a date which is earlier than known elsewhere in the world.

Our evidence is found at the southeast end of Huon Peninsula (Fig. 1), where a flight of raised coral terraces preserves the former coastal environment of a succession of Upper Pleistocene stages. The terraces have been described and dated by ^{14}C and $^{230}\text{Th}/^{234}\text{U}$ methods, showing continual tectonic uplift of the coast^{8–11}. Each coral reef terrace formed when a glacio-eustatic rise of sea level overtook the rising land⁹. During each period of reef formation, the coastal environment included local lagoons and fringing reefs. By analogy with the present coastal fringe with its Holocene reefs, these provided a productive and hospitable habitat for human occupation^{9,12}. Figure 1 shows the terrace flight in the vicinity of the archaeological site described here.

The archaeological site occurs in a stream gully running into a small palaeo-lagoon on the reef complex labelled IIIa by Chappell⁹, which is dated as 45,000 to 53,000 years by $^{230}\text{Th}/^{234}\text{U}$. This age and the $^{230}\text{Th}/^{234}\text{U}$ chronology of the entire terrace sequence is supported by correlation with deep-sea core records¹³. In the area shown in Fig. 1, reef IIIa overlies seaward-dipping bedded sandy tufts that are exposed in several streams and are seen to pass inland beneath the front of the next reef higher in the sequence. A set of three strongly weathered tephra occur in the depression behind reef IIIa and on the lower front of the rise to reef IVb (Fig. 1). Artefacts found in the excavation described below occur within the tephra sequence. The tephra are considerably altered by pedogenesis. All are clay-like and the upper two are dark brown to black, and each contains fine sand to fine silt-size angular tephric quartz in greater concentrations towards their bases. The tephra pile varies from 1.5–2.8 m thick due to slope wash and redeposition after each fall, as well as to downslope creep which is indicated by small slip planes visible in the excavation.

The age of the tephra is critical to our conclusions about the age of the artefacts. The tephra do not occur on 6,000-year-old (Holocene) reef I, which is broad and contains potential tephra-trapping palaeo-lagoons (Fig. 1). They have not been found on reef II, which is dated to 28,000 to 30,000 years^{9,11}, nor on reef IIIb which is 40,000 years old^{9–11}, although the possibility they

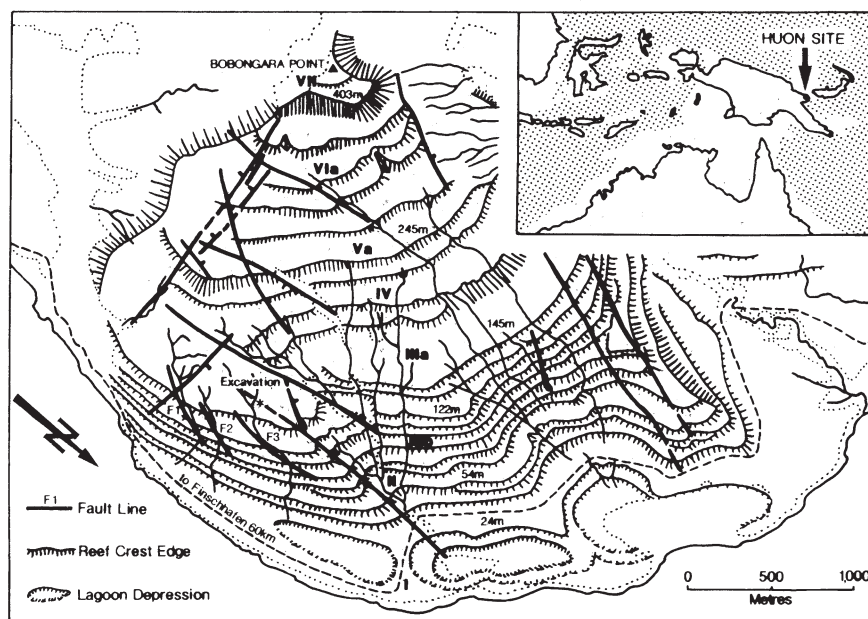


Fig. 1 Bobongara area of Huon Peninsula coral terraces. Archaeologic site marked * Excavation. Inset shows land exposed at times of Pleistocene low sea levels (white areas), and location of Huon Peninsula site in Papua New Guinea.

were on these reefs and subsequently were stripped cannot be eliminated. The inference that the tephrae are older than 40,000 years is supported by thermoluminescence (TL) dating of the quartz particles within each unit. This dating was done at Australian National University, using the fine-grain technique¹⁴. All samples show well-defined TL plateaux from about 350° to 475 °C. Laboratory-induced TL growth curves were linear, and fading tests (1 month) found no TL loss. Hence, we are confident of the calculated archaeologic doses. Dose rate, based on laboratory analyses of potassium (K) and total α count rate for each tephra, is less certain due to a peculiarity in the K-values. Measured K_2O content of the tephrae ranges from 0.05–0.11%. This is very low for tephra, and K-leaching is suspected to have occurred during strong pedogenesis. The nearby Bismark volcanics average K_2O of 1.2%¹⁵. Hence, dose rates are estimated on the basis of (1) measured K_2O , and (2) $K_2O = 1.2\%$. The latter is almost certainly greater than the average value over the history of the deposits. The alpha dose rate is taken as measured, which may also be higher than the historical average due to thorium growth in the neighbouring reefs, which are in U-series disequilibrium^{8,10}.

TL data and calculated ages are shown in Table 1. Ages based on measured K_2O and assumed K_2O (=1.204%) are both consistent with stratigraphic order, showing that T1 is significantly younger than T2 and T3, which are similar to each other. Ages based on measured K_2O are higher than those based on assumed K_2O because the K_2O values are lower. We believe that ages based on assumed K_2O are almost certainly too low, first because they are based on much higher K_2O than measured, and second because the assumption that $H_2O = 0$ is unrealistic as annual

rainfall at the site is 2,500 mm and the soil has been found to be generally wet during excavation. Soil moisture reduces dose rate, and the zero H_2O assumption leads to unduly low age estimates. We conclude that the T2 and T3 are at least 40,000 years old, and probably T1 as well, consistent with their occurrence on Reef IIIa but not on the younger reef terraces.

Stratigraphy of the archaeological excavation is shown in Fig. 2. The site is adjacent to an ephemeral channel (Jo's Creek) which descends the front of reef IVb and crosses the depression behind the crest of reef IIIa. Two sections were excavated, one along the southeast creek bank and the other at right angles to this ('trench section' in Fig. 2). The creek section was excavated as a vertical face cut slowly back into the slope from the channel, and the trench was cut slowly downwards maintaining a horizontal floor until basal tuff and limestone was encountered. Sieving was not possible in the sticky clay-like tephrae, and most work was by trowel. Figure 2 shows that the tephrae are continuous but vary in thickness. Some variation is due to mass creep, as mentioned, and some reflects infilling of temporary channels which are thought to have formed by runoff before and between tephra falls. Such a channel is seen beneath findspot 2 in the trench section (Fig. 2).

Artefacts were recovered at several points indicated in Fig. 2, the most important being at the interface between T2 and T3 at findspots 1 and 2. These and that from findspot 3 are waisted axes, described shortly. A stone core and 2 flakes were found at and near findspot 4. All were recovered from *in situ* positions by direct excavation. The waisted axe at findspot 2 was in several pieces with interstitial tephra T2, attesting to burial after breakage with little subsequent displacement. The waisted axe from

Table 1 Thermoluminescence dating results

Tephra unit	Archaeological dose (rad)	Measured K_2O	TL age (yr BP)			
			Minimum ADR (rad yr ⁻¹)	Age*	Maximum ADR (rad yr ⁻¹)	Age
T1	12,600	0.0543	0.299	42,100	0.402	31,300
T2	9,430	0.1076	0.156	60,500	0.254	37,100
T3	8,732	0.1372	0.146	59,800	0.241	36,200

Column 5 shows ages based on measured K_2O and column 7 ages based on assumed $K_2O = 1.204\%$. Other assumed parameters are $Rb = 110$ ppm, $H_2O = 0$, and cosmic dose = 0.015 rads yr⁻¹. Standard errors of measurement are not shown as these are much less than K_2O uncertainty. Tephra units are labelled T1 to T3 in descending stratigraphic order.

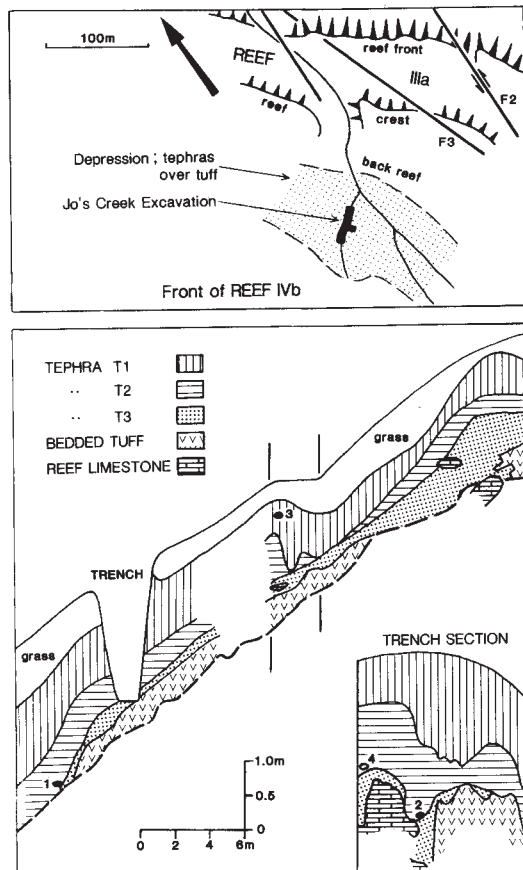


Fig. 2 Location of excavation (Jo's Creek) on Terrace IIIa at Bobongara, and stratigraphic sections at excavation. Main section follows creek; trench section at right angles to this. Numbered spots show locations of artefacts described in text.

spot 1, shown in Fig. 3a, is heavy (1.68 kg) and is marked by a distinct groove between the waisting notches. It is made of 2-pyroxene andesite comparable with boulders in the nearby Masaweng River. It has a skin of semi-opalline cristobalite (X-ray diffraction determination) and a thin section from a 1-cm core from the underside shows the albitic groundmass to be altered to microcrystalline quartz. These features are consistent with long burial in a weathering tephric environment. We refer to the tool as a waisted 'axe' on the grounds of its similarity in terms of size, weight, strength, and edge characteristics to axes. We interpret the groove across the waist as due to haft-lashing, by analogy with ethnographic examples from Melanesia¹⁶ and Australia.

In addition to the artefacts found *in situ*, over 100 waisted axes of similar dimensions and pattern, although without hafting grooves, were found loose in creek beds around the site, mostly on or somewhat above reef IIIa. All are unifacial, of similar material to that in Fig. 3a, apparently manufactured by spallation from rounded river boulders followed by flake retouching, often very steep, of the flake-scar surface. A typical example is

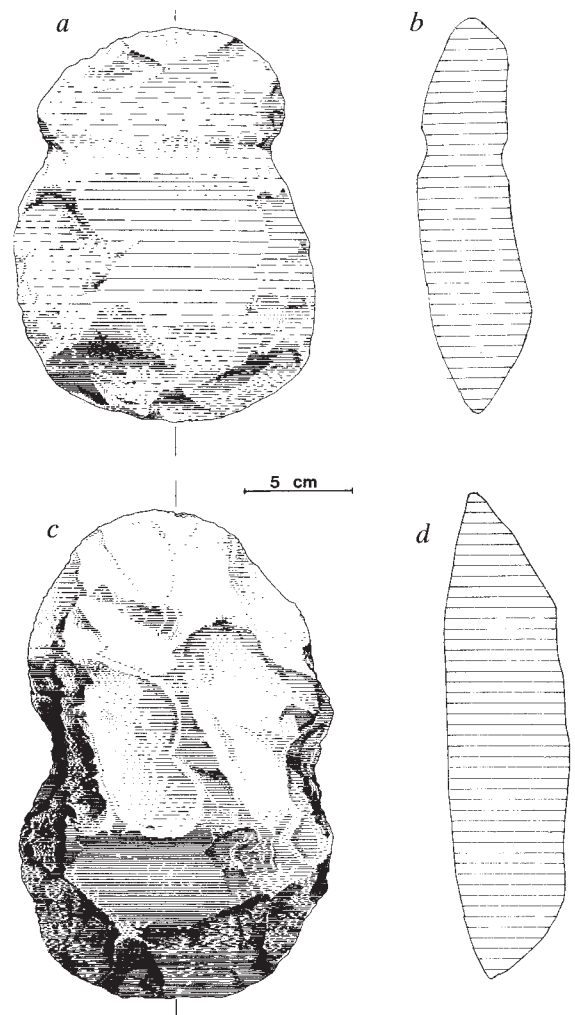


Fig. 3 a, b, Grooved waisted axe from findspot 1 in excavation. c, d, Typical non-grooved waisted axe from near to excavation.

shown in Fig. 3b. Other loose artefacts in creeks at or above the site include a chert core, various flakes, and one 'stemmed' and one 'knobbed' axe, both unifacial and made of andesite. Our waisted axes have similarities with artefacts discovered elsewhere. In highland New Guinea, waisted axes at 26,000 years are reported at Kosipe⁴ and occur from 6,000 to over 10,000 BP at Yuku¹⁷. Pleistocene ages are inferred for waisted axes at Kangaroo Island in south Australia and at Mackay in north Queensland¹⁸. Waisted axes of inferred Holocene age occur at Botal Tobago¹⁹ and Japan²⁰, in east Asia, although these appear to be smaller than the Huon Peninsula tools.

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Reproductive failure in common seals feeding on fish from polluted coastal waters

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The population of common seal *Phoca vitulina* in the westernmost part of the Wadden Sea, The Netherlands, has collapsed during the past few decades^{1,2}. Between 1950 and 1975 the population dropped from more than 3,000 to less than 500 animals. Comparative studies of common seal populations from different parts of the Wadden Sea reveal that pup production has declined sharply only in the western (Dutch) part²⁻⁴. A comparative toxicological study⁵ on the levels of heavy metals and organochlorines in tissues of seals from the western and northern parts of the Wadden Sea shows that only the polychlorinated biphenyl (PCB) levels differ significantly. This is predominantly a result of PCB pollution from the river Rhine⁶⁻⁹, which mainly affects the western (Dutch) part. PCBs are thought to be responsible for the low rate of reproduction in Dutch common seals on the basis of epidemiological and experimental data on the ability of PCBs to interfere with mammalian reproduction^{5,10}. Here I report that reproductive failure in common seals from the Dutch Wadden Sea is related to feeding on fish from that polluted area. This is the first demonstration of a causal relationship between naturally occurring levels of pollutants and a physiological response in marine mammals.

An experiment with two groups of 12 female common seals was carried out to test the detrimental effects of PCBs on seal reproduction. Each group, consisting of 7 seals from the east coast of the United Kingdom and 5 seals from the Museum of Natural History at Texel, was fed a diet containing different levels of pollutants. Group 1 received fish (predominantly plaice, flounder and dab, with some eelpout and hooknose) caught in the western part of the Wadden Sea. Group 2 received fish

(mainly mackerel) from the north-east Atlantic. The fish were maintained at -28°C and thawed before being fed to the seals. The diets are comparable with respect to nutritional quality, except for fat levels; this was compensated for by a high daily intake. Residue analysis for aldrin, dieldrin, endrin, heptachlor, heptox, α,β,γ -hexachlorocyclohexane, pentachlorobenzene, hexachlorobenzene, *pp'*-dichlorodiphenyl-dichloroethylene (DDE), *op'*-dichlorodiphenyl-dichloroethane, *pp'*-dichlorodiphenyl-dichloroethane and PCBs showed statistically significant differences between the two diets for PCBs and *pp'*-DDE. The average daily intake (during ~ 2 years) was 1.5 mg PCBs and 0.4 mg *pp'*-DDE for group 1, and 0.22 mg and 0.13 mg for group 2 (J. P. Boon, P.J.H.R., J. Dols, and P. F. Wensvoort, unpublished data). Three males receiving Atlantic fish were alternated between both groups during the mating period. To detect whether hormonal regulation was affected by pollutants, blood samples were taken regularly and serum concentrations of progesterone and oestradiol- 17β were determined. Because the timing of oestrus differs for individuals, all profiles have been synchronized by taking the day of previous delivery as day zero and adjusting the profiles of non-pregnant animals accordingly. Although there is some published information about the hormonal changes during reproduction in seals^{11,13}, none of these studies reported the changes during a complete annual reproductive cycle in a specific group of females. The oestradiol- 17β and progesterone concentration profile of the control group (Fig. 1e,g) illustrate the normal annual cycle. The number of non-pregnant animals in the control group is too small to consider their reproductive cycle as 'normal' for non-pregnant females.

No statistically significant differences (Spearman, $p < 0.005$) were found in the progesterone and oestradiol- 17β profiles of pregnant seals from groups 1 and 2. Identical tests for the non-pregnant animals in both groups gave the same result. This is important as it implies that the differences in diet do not influence hormone patterns. The patterns resemble those for eutherian mammals in general^{14,15}. Pinnipeds are classified as having an obligate and seasonal embryonic diapause^{16,17}. In common seals in this study implantation probably occurred at

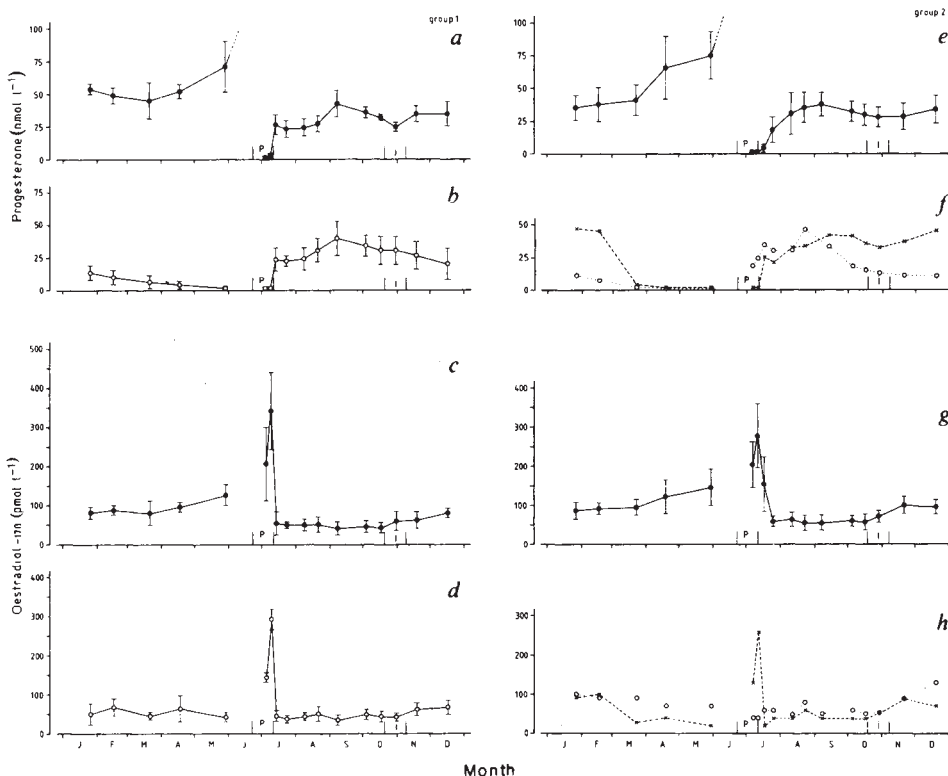


Fig. 1 Circulating serum concentrations of progesterone and oestradiol- 17β during the reproductive season 1983-84, in common seals feeding on fish from the Wadden Sea (group 1) and on Atlantic fish (group 2). a-d, Group 1 (a,b,c,d): (●---●), pregnant seals ($n=4$); (○---○), non-pregnant seals ($n=8$). e-h, Group 2: (●---●), pregnant seals ($n=10$); (×---×) and (○---○) non-pregnant seals. Vertical bars, 1 s.d.; p = period over which previous parturitions occurred; i = implantation period. Blood samples were obtained from veins in the hind flippers after restraining the animals on a V-shaped bench. The blood (~ 8 ml) was collected in vacutainers, centrifuged and the serum stored frozen at -20°C . Serum concentrations of progesterone and oestradiol- 17β were determined by radioimmunoassay. The intra run coefficients of variation for progesterone and oestradiol- 17β were consistently below 3.0% and 3.05% respectively.

the end of October or beginning of November, and was characterized by an increase in oestradiol-17 β levels (Fig. 1, c, g).

The reproductive success was significantly lower in group 1 than in group 2 (Table 1; $p < 0.02$, Fisher's exact probability test). The hormone profiles of the non-pregnant animals in group 1 show that the effect occurs at a stage of the reproductive process around implantation; the follicular, luteal and post-implantation phases until the end of gestation are not affected. These findings corroborate the results from experiments with mink^{18,19} where PCBs impaired reproduction: ovulation, mating and implantation occurred but were followed by early abortion or resorption. These results may be due to hormonal disturbance, to direct dominant-lethal action or to an embryo lethal effect caused by toxicants. Hormonal disturbance may be caused by disruption of the steroid synthetic pathway resulting in reduced circulating levels of hormones. PCBs are known to cause microsomal enzyme induction, accelerating hydroxylation of body steroids such as oestrogens⁵. To determine if pollutants suppress circulating levels of hormones during the implantation period, the four subgroups (pregnant and non-pregnant seals in groups 1 and 2) are compared. A statistical test revealed no difference between progesterone levels of pregnant animals in group 2 and non-pregnant animals in group 1. It is concluded that progesterone levels were not reduced in group 1. The rise in oestradiol levels of the non-pregnant seals in group 2—indicating follicular growth—is lacking in non-pregnant seals of group 1, which suggests that the nature of non-pregnancy differs between the groups. Because there were only 2 non-pregnant animals in group 2, this could not be tested statistically. The results also demonstrate that the levels of oestradiol in all group 1 seals are lower than those in group 2 combined, ($p < 0.05$, Wilcoxon), although the initial rise in some of the animals of group 1 (Fig. 1c) was apparently sufficient to ensure reproductive success. The mechanisms behind the smaller increase in oestradiol levels and its consequences for the priming effect on the endometrium, as well as the maternal rejection response²⁰⁻²², will be discussed in detail elsewhere. Hypotheses about impaired steroid binding capacity by PCBs²³, a dominant-lethal action and an embryo lethal effect cannot be tested with the information available.

I conclude that the reproductive success of the seals receiving the diet with the highest level of pollutants was significantly decreased. No circumannual reduction in levels of circulating hormone levels was observed. The reproductive process is disrupted in the post-ovulation phase. The period around implantation seems to be the most sensitive stage, but no conclusions about the mechanism of action can be drawn. A similar experiment was simultaneously carried out with the American mink *Mustela vison*, a fish-eating mammal with a comparable reproductive physiology. It was designed to test whether pure PCBs had the same effect as the PCB-polluted fish. Three groups of 'Standard' type mink were fed either a basic diet of commercial mink cereal, or the same basic diet supplemented with livers from fish caught in the western part of the Wadden Sea or with Clopen A-60 or A-30. The results show that reproduction is inhibited at very low (25 μ g per day) levels of PCB intake and that the effects of the pure PCB diet were identical to those of contaminated fish diet¹⁸.

Irrespective of the precise mechanism involved, the results from this study show that the reproductive failure in common seals from the Dutch Wadden Sea is related to feeding on fish from that polluted area. The available epidemiological experimental data on effects and levels of PCBs in seals and mink fed on fish from this area^{5,27} suggest that these organochlorines are the main cause of this failure.

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Table 1 of this paper appears on page 418.

A common mammalian plan of accessory optic system organization revealed in all primates

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The accessory optic system (AOS), which was described as early as 1870 by Gudden¹, constitutes a distinct midbrain visual pathway in all classes of vertebrates². In non-primate mammals, retinal fibres of this system project to a set of three nuclei^{3,4}: the dorsal (DTN), the lateral (LTN) and the medial (MTN) terminal nuclei. Whereas all AOS cells respond to the slow motion of large visual stimuli, the neurons are tuned to complementary directions of movement⁵: horizontal temporo-nasal direction for the DTN, vertical up and down for the LTN and vertical down for the MTN. It has thus been suggested that these nuclei establish a system of retinal coordinates for the detection of whole field motion⁶. As the AOS provides direct and indirect pathways to both oculomotor and vestibular structures^{7,8}, each of these nuclei is thought to be an essential link in the co-ordination of eye and head movements in relation to movement within the visual field. One problem for the generalization of this theory is that the medial terminal nucleus has never been found in primates. In this report we establish both the existence of this nucleus and its afferent input from the retina in all major groups of primates (prosimians, New and Old World monkeys and apes), indicating a common anatomical plan of organization of the AOS in mammals.

We used both autoradiographic and histochemical anterograde tracing techniques to study the retinal projection to the AOS in the two primate suborders⁹ Strepsirhini (prosimians: lemurs and lorises) and Haplorhini (simians: New and Old World monkeys, apes and man). For the Strepsirhines we studied five mouse lemurs (*Microcebus murinus*) and one bushbaby (*Galago demidovii*). Haplorhine species included two marmosets (*Callithrix jacchus*), three macaques (*Macaca fascicularis*) and two gibbons (*Hylobates concolor*). Most animals were injected with a radioactive amino acid mixture (500-

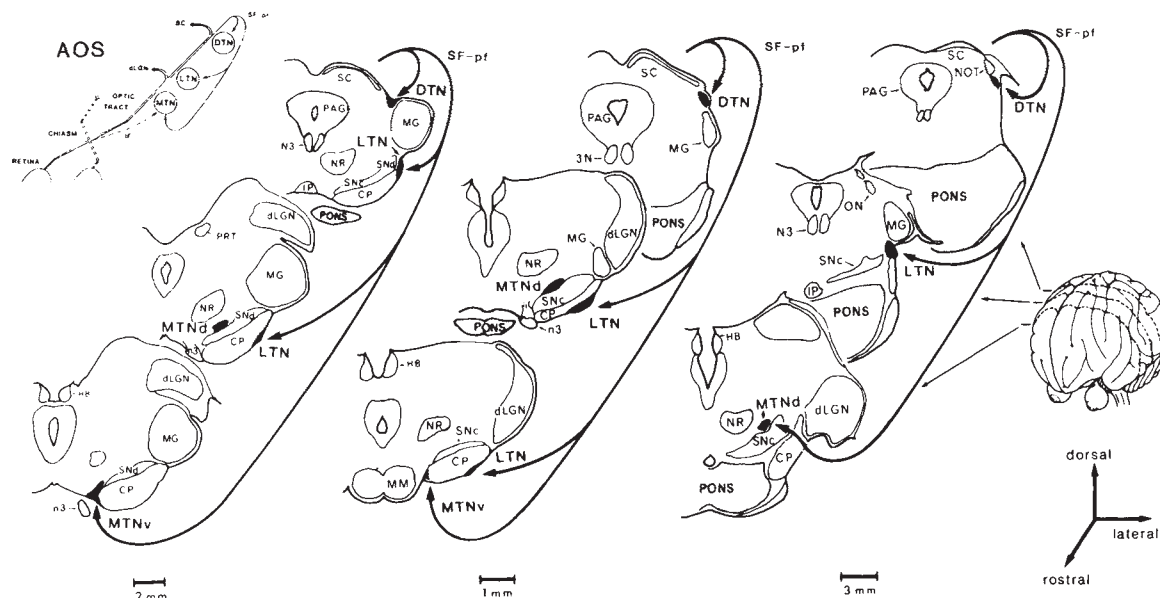


Fig. 1 Organization of the accessory optic system compared in a non-primate (cat), a strepsirrhine primate (mouse lemur) and a haplorhine primate (gibbon). Animals received an intraocular injection of radioactive amino acids to reveal the distribution of retinal fibres and terminals. Drawings, scaled to the same size, are from coronal sections (lower right: approximate section levels shown on brain of gibbon) showing the relative positions of the three terminal nuclei (MTN, LTN, DTN, dark shading) of the AOS. Arrows, course of the superior fasciculus (SF-pf) (the main component of the AOT). As shown in the simplified schema in the upper left, this fasciculus separates from the main optic tract in the region of the brachium of the superior colliculus and then courses rostrally. The AOS in strepsirrhine primates is similar to that of other mammals, but the MTNv is reduced in size. In haplorhine primates a retinal projection to the MTNv is lacking, and AOS fibres show a shortened trajectory through the cerebral peduncle to the MTNd. The extensive rostral development of the pons and pyramidal tracts in primates suggests that the reduction of the MTNv in Strepsirhini and its absence in Haplorhini is the consequence of a resulting morphological reorganization in the anterior region of the midbrain. AOS, accessory optic system; CP, cerebral peduncle; dLGN, dorsal part of the lateral geniculate nucleus; DTN, dorsal terminal nucleus of the AOS; HB, habenular nucleus; IF, inferior fasciculus; IP, interpeduncular nucleus; LTN, lateral terminal nucleus of the AOS; MG, medial geniculate nucleus; MM, mamillary nucleus; MTNd, dorsal division of the medial terminal nucleus of the AOS; MTNv, ventral division of the medial terminal nucleus of the AOS; n3, oculomotor nerve; N3, oculomotor nucleus; NOT, nucleus of the optic tract; NR, red nucleus; ON, olivary pretectal nucleus; PAG, periaqueductal gray; PRT, pretectum; SC, superior colliculus; SF-pf, posterior fibre branch of the superior fasciculus; SNc, substantia nigra, pars compacta; Snd, substantia nigra, pars diffusa.

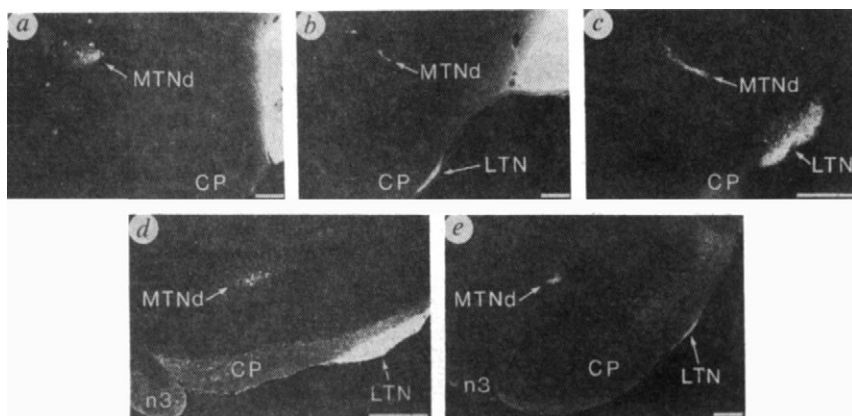


Fig. 2 Distribution of anterograde label transported from retinal ganglion cells to the MTNd (and the LTN) in: *a*, gibbon; *b*, macaque; *c*, marmoset; *d*, lemur; *e*, cat. The microphotographs of these coronal sections are viewed under dark-field illumination. Note the similar topographic location of the MTNd medial to the cerebral peduncle and the substantia nigra (towards upper left) in all species. Scale, 500 μ m. CP, cerebral peduncle; LTN, lateral terminal nucleus of the accessory optic system; MTNd, dorsal division of the medial terminal nucleus; n3, oculomotor nerve.

1,000 μ Ci of 3 H-proline and 3 H-leucine) in the posterior vitreal chamber of the eye. After survival times of 24–60 hours the brains were processed according to the usual autoradiographic procedures for frozen or paraffin sections. In other primates, the eye was injected with 10–20 μ l of a 40% solution of the anterograde tracer horseradish peroxidase (HRP; Sigma type VI) dissolved in sterile saline with 2% DMSO. Survival time was 36–60 hours. Frozen sections of the brains from these animals were treated with the tetramethylbenzidine method¹⁰. Brains from each series were sectioned in either frontal, sagittal, or horizontal planes and stained with cresyl violet to study cytoarchitecture. Retinal projections to the AOS were compared in species from eight other orders of mammals: Carnivora, Rodentia, Chiroptera, Dermoptera, Hyracoidea, Pholidota, Marsupialia and Edentata.

In all mammalian species studied, fibre tracts and the three terminal nuclei of the AOS were labelled by anterograde transport of the neuronal tracer from retinal ganglion cells. The majority of retinal fibres that form the accessory optic tract (AOT) cross in the optic chiasma, although a small component remains uncrossed. The AOT diverges from the main optic tract at the level of the brachium of the superior colliculus. The tract becomes visible on the superficial aspect of the brainstem as it retraces a rostral pathway to the anterior base of the midbrain¹¹, terminating in each of the AOS nuclei along its course (Fig. 1).

The MTN is the most conspicuous of the three AOS nuclei in non-primates and can be divided into a ventral division (MTNv) located at the ventromedial base of the cerebral peduncle, and a dorsal division (MTNd) extending into the region of the substantia nigra^{3,12}. In primates, a retinal projection

to the ventral division was observed only in Strepsirhines but the dorsal division receives retinal terminals in all species studied.

The projection to the MTNd is remarkably similar in primates and non-primates (Fig. 2). It is located in a similar position in all species (medial to the substantia nigra pars compacta and ventro-lateral to the medial lemniscus and the red nucleus). The distribution of terminal label (silver grains or HRP granules) in the MTNd is often reticular in appearance, as synaptic contacts of retinal axons are mainly situated on dendrites rather than on the cell bodies¹³. Fibres of the AOT course to the nucleus by penetrating directly through the cerebral peduncle, forming a characteristic obliquely-oriented fibre bundle in the region of the substantia nigra.

In some primates (*Macaca*, *Callithrix*) the MTNd is rather small, whereas in the gibbon (*Hylobates*) the nucleus extends rostrocaudally in the midbrain for >2.0 mm. The MTNd is partially embedded within the substantia nigra and in some instances the cells of these two structures actually intermingle. Several cytological features allowed us to distinguish the cells of each nucleus, however. The MTNd neurons are typically pale stained, and ovoid or fusiform in shape. In contrast, nigral cells are comparatively large sized, polygonal shaped, and intensely stained with cresyl violet; they also have a large clear cell nucleus and a dense compact nucleolus. Depending on the species, the two populations of cells are also distinguished by additional features. For example in the gibbon, as in other apes and man, the cytoplasm of nigral cells is densely packed with black granules of melanin¹⁴. These cells show an endogenous red fluorescence under dark-field illumination due to the presence of melanin whereas the MTNd cells, which lack pigment, do not fluoresce. Anterograde label was distributed only over the cells of the MTNd.

In the strepsirhine primates *Microcebus*¹⁵, *Galago*¹⁵ and *Nycticebus*¹⁶, there is also a small but distinct ventral division of the MTN, which receives retinal afferents. As in other mammals, such as the cat, the MTNv is located at the base of the cerebral peduncle, immediately rostral to the emergence of the oculomotor nerve. The cells in this part of the MTN are densely stained, small and round in shape (average diameter 14 µm), and thus differ from those of the dorsal division, which are highly elongated (typically 8 µm × 40 µm). The cytoarchitecture and pattern of retinal projections to the two subdivisions of the MTN in *Microcebus* closely resemble those of the cat. In the cat the ventral and dorsal subdivisions clearly form a topographically continuous nucleus, whereas in *Microcebus* these two parts are interconnected by a few fine fibres. In haplorhine primates we could not identify either terminal label or a cell group typical of the MTNv.

The absence of a distinct ventral portion of MTN in haplorhine primates has been interpreted as the result of a simple regression of the nucleus during primate evolution^{17,18}. However, we suggest that the neurons of the MTNv have migrated in position to a more dorsal location and form a single constituent nucleus with the MTNd. Such a dorsal shift of the MTNv in primates could be a consequence of the extensive development of the pyramidal tract¹⁹ (related to precise motor control of the limbs), resulting in a rostral extension of the pons below the cerebral peduncle which influences the topographical relations between the nuclear groups in this region. Since in other mammals the neurones of the ventral and dorsal divisions of the MTN differ in cytoarchitecture¹², efferent connections^{7,8} and histochemical properties¹², the hypothesis of a dorsal topological shift of the MTNv in primates could be experimentally verified if the same distinctions exist in this single constituent of the MTN.

Our results unequivocally demonstrate that in primates at least one subdivision of the MTN is present and receives a monosynaptic retinal projection. This conclusion is supported by the presence of retinal terminals to a well defined cellular

aggregate located in a homologous region of the midbrain tegmentum and by the typical course of accessory optic fibre tracts to the nucleus. Preliminary results²⁰ also provide evidence that, as in the rat^{7,8}, the rabbit^{7,8} and the cat²¹, the MTN in *Macaca* also sends an efferent projection to the vestibular nuclei. Thus the intrinsic organization of the AOS and its relations with other sensory systems are constant features in all mammalian species.

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A cell surface molecule distributed in a dorsoventral gradient in the perinatal rat retina

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Brain topography may have its earliest expression as spatial gradients of molecules controlling the deposition of neurones and neuronal processes^{1,2}. In the vertebrate visual system there is evidence that the stereotyped alignment of central retinal projections relies on an initial spatially organized distribution of molecules in both the retina and its central target nuclei³⁻⁶. We used an immunological approach to look for molecules that are so organized and produced a monoclonal antibody (JONES) which shows a pronounced dorsal to ventral gradient of binding in the rat retina throughout the period when retinal ganglion cell axons are forming topographically organized projections within the central nervous system (CNS). Binding is present throughout the radial thickness of the retinal epithelium in regions where post-mitotic neurones are generated but is not associated with any consistent histological characteristic of the tissue. The antibody was shown to bind on the cell surface of freshly dissociated retinal cells, and dorsal retinal quadrants were found *in vitro* to have nearly twice as much antigen as ventral retinal quadrants. Initial biochemical characterization of the target epitope reveals that it is a lipid present in chloroform/methanol extracts from perinatal retina and is sensitive to neuraminidase digestion.

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Fig. 1 Immunocytochemical distribution of JONES antibody binding in retinas sectioned parallel to their dorsoventral axes. *a*, Staining of central retina in the E13 eye; *b*, an adjacent 'blank' section reacted with second antibody alone; *c*, the dorsoventral gradient in the E17 eye. Arrow in dorsal retina, the initial expression of the antigen in the region adjacent to the zone at the ciliary margin that still contains only proliferating cells; double arrow, JONES binding in the optic fibre layer of both dorsal and ventral retina; *d*, an adjacent section counterstained with toluidine blue. Scale bars *a*–*d* = 150 μ m; *e*, the retinal epithelium at P0; JONES binding is now distributed throughout the epithelium but a dorsoventral gradient is still apparent; *f*, adjacent 'blank' section. Scale bar *e*, *f* = 500 μ m. In the P0–P3 retinas, the zone of neuroblast proliferation extends to the ciliary margin of the eye^{7,8}. JONES binding also extends to the ciliary margin at these ages; *g*, higher magnification of the dorsoventral difference in JONES binding in the P0 eye. Horizontal sections of eyes at equivalent stages of development fail to show this pronounced anisotropy in JONES binding; *h*, the equivalent region of another P0 eye from a pup pulsed with ³H-thymidine (1 μ Ci per g body wt., NEN, specific activity 6.7 Ci mmol⁻¹) two hours before it was killed. The most vitreal border of the proliferative zone in central retina is delineated by high grain densities over nuclei synthesizing DNA, post-mitotic amacrine and ganglion cells are present in inner retina. No differences between dorsal and ventral retina are apparent. Scale bar *g*, *h* = 50 μ m.

Methods. The JONES antibody was obtained by inoculation of a BALB/c mouse with retinal epithelia from fetal rats killed 14–21 d after sperm positive vaginal flushes, (E13–E20) rats. The mouse received three intraperitoneal injections of a crude homogenate of 6–10 lightly fixed retinas (2% paraformaldehyde for 30 min). For the first inoculation the retinal homogenates were suspended in complete Freund's adjuvant. Incomplete Freund's was used for the subsequent inoculations spaced two weeks apart. A final boost of ~5 retinas homogenized in phosphate buffered saline (PBS) was given intravenously four d before the fusion of spleen cells with P3-NSI/1-Ag4-1 plasmacytoma cells^{33–35}. Screening of antibodies was performed with indirect immunofluorescence using tissue culture supernatants and sections of retina in which E13–E14 retinal epithelia had been nested inside P1 eyes. The JONES monoclonal antibody was sub-cloned three times by limiting dilution and shown by Ouchterlony analysis with subclass-specific sera to be immunoglobulin class M (IgM). Its binding has been examined using indirect immunofluorescence with goat anti-mouse IgG (H + L) (Cappel) or silver-intensified colloidal gold staining using a specific goat anti-mouse IgM (Janssen). Both procedures gave a staining pattern in the form of puncta that appeared to outline the perimeter of cell bodies. The retinal immunocytochemical staining above uses the silver intensified immunogold procedure in dark field illumination. Tissue was fixed overnight at 4°C, cryoprotected in 30% sucrose in PBS and aligned in OCT mounting medium (Tissue-Tek, Miles) either as a whole fetal head or with the dorsal retinal pole indicated by a single remaining flap of skin. Sections were cut at 10 or 15 μ m on a Hacker cryostat.

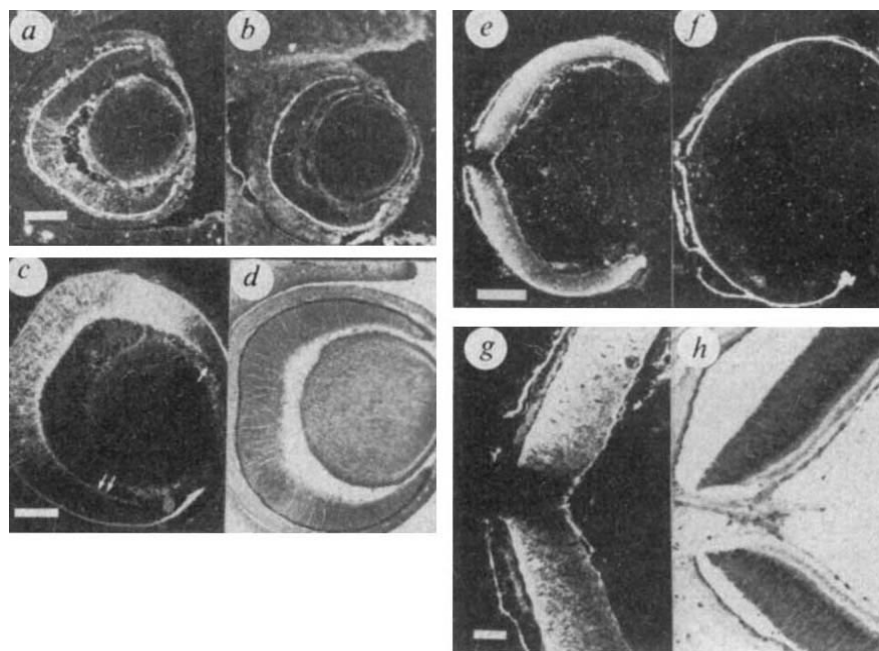
The JONES antigen was first detected in the rat at embryonic day 12 or 13 (E12–E13) where it was localized to central retina (Fig. 1*a*). By E17–E18 binding was observed along regions of the vitreal surface of the retina next to where an optic fibre layer had formed. In cellular regions of the epithelium the antigen was clearly distributed in gradient (Fig. 1*c*). JONES binding was invariably high in dorsal retina, decreasing gradually in more ventral retinal regions. In the E16–E17 rat a zone of postmitotic amacrine and ganglion cells is apparent in all but the peripheral rim of retina^{7,8}. In dorsal retina the JONES antigen was expressed close to the boundary where these post-mitotic cells first appeared (Fig. 1*c*, *d*; arrows), but in ventral retina expression decreased to background levels well within the zone of post-mitotic ganglion and amacrine cells. The dorsal to ventral gradient of JONES binding was retained in the post-natal day 0 (P0) eye (Fig. 1*e*), and was still apparent, though less pronounced, at P3. Throughout the E17–P3 period ventral retina was indistinguishable from its dorsal counterpart on the basis of histological criteria or thymidine uptake (Fig. 1*d*, *g*, *h*).

Cells freshly dissociated from the neonatal eye expressed the JONES antigen on their surfaces (Fig. 2). Differences in JONES binding to different retinal quadrants were subsequently quantified using an *in vitro* assay (Fig. 3*a*). The small size of the early rodent eye precluded reliable dissections of the retina into subquadrant pieces and quantification of the absolute amount of binding was difficult because the degree to which the antibody penetrated the full thickness of the epithelium was unknown. Consequently the results of this assay do not give the maximal

differences in antigen concentration between dorsal and ventral retina, but even so JONES binding was found to be ~1.5 $\times$ higher in dorsal than in ventral retinal quadrants (Fig. 3*b*). This shows that the immunocytochemical differences in JONES binding do not result from fixation artefacts or variations in tissue mass.

Binding of JONES to aldehyde-fixed sections of late fetal and neonatal retina was removed by pretreatment of the fixed tissue with organic solvents and detergents, suggesting that the molecule recognized was a lipid. No antibody binding was observed on Western blots of late fetal or neonatal retinal extracts. Figure 4*a* illustrates immunoblots from thin layer chromatographic (TLC) plates of chloroform/methanol extracts of P4 retinas run in a chloroform:methanol solvent system. When such extracts were partitioned into aqueous and organic phases, all the immunoreactivity was found in the aqueous phase as a discrete band that migrated slightly below ganglioside G_{M2}. A single band of immunoreactivity was also found when the TLC plates were developed in a 1-propanol:aqueous calcium chloride solvent system (data not shown). No immunoreactivity suggestive of binding to lipoproteins or phospholipids was observed at the origin or in the upper regions of these lanes. The immunoreactive band was eliminated by treatment of the retinal extracts with neuraminidase (Fig. 4*b*). These results imply that the JONES epitope is carried on a relatively simple polar lipid, probably a minor species of ganglioside.

Position-dependent cell surface differences between dorsal and ventral retinal cells are suggested by preferential adhesion



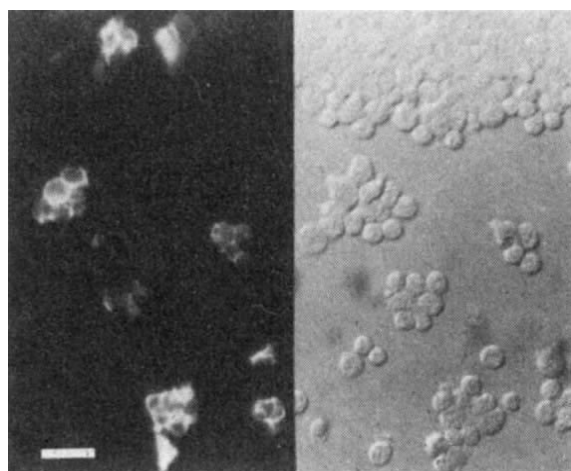
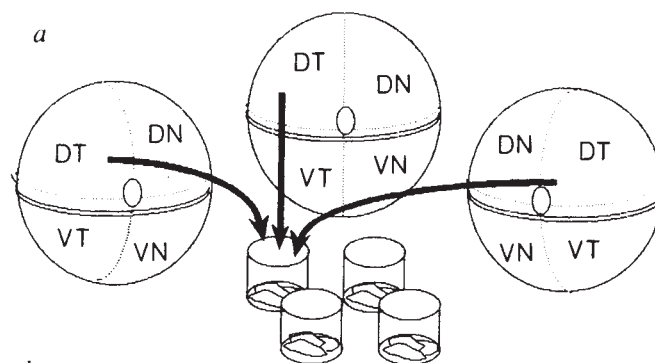


Fig. 2 Cells freshly dissociated from P2 retinal epithelia incubated with the JONES monoclonal antibody and then with rhodamine-labelled goat anti-mouse second antibody. Left, epifluorescent photomicrograph showing JONES labelling on the plasma membranes of cells; right, interference contrast photomicrograph of the same field. Scale bar = 20 μ .

Methods. Twenty P2 retinas were incubated for 20 min at 37 °C in 0.01% trypsin (Gibco 1:150), washed in PBS, titrated in PBS with DNase (5 μ g ml⁻¹), centrifuged for 10 min, resuspended in 1 ml PBS and incubated for 1 h with the IgM fraction of JONES ascites fluid (1:100). The cells were then washed 3 times in PBS by centrifugation, resuspended in 1 ml PBS, incubated for 1 h at room temperature in goat anti-mouse IgG (1:50, H + L, Cappel), washed 3 times in PBS by centrifugation, pipetted onto a slide, covered and photographed immediately.



b

	DN c.p.m.	DT c.p.m.	VN c.p.m.	VT c.p.m.	D/V ratio	N/T ratio	DN/VT ratio	DT/VN ratio
1	3,393	5,315	2,856	2,799	1.54	0.77	1.21	1.86
2	5,877	3,818	3,451	3,217	1.45	1.33	1.83	1.11
3	3,918	2,710	2,674	2,130	1.38	1.36	1.84	1.01

Fig. 3 *a*, Diagrammatic representation of the assay for differential JONES binding to P3 retinal quadrants; *b*, the results of three replicates of this experiment using the quadrants from three retinas per assay point D, dorsal; V, ventral; N, nasal; T, temporal retinal halves. The difference between the summed counts for both dorsal and both ventral retinal quadrants was highly significant ($P < 0.0025$) by paired sample Student's *t*-test. Control experiments without JONES antibody or with other known non-reactive IgM antibodies revealed only low levels of binding and no differential binding to the retinal quadrants.

Methods. A large blood vessel running approximately horizontally along the back of the eye immediately ventral to the optic nerve head was used to distinguish dorsal from ventral retina. A cut perpendicular to this vessel running through the optic nerve distinguished nasal from temporal retinal halves. The pigment epithelium and inner limiting membrane were dissected from each retinal quadrant. The corresponding quadrants from three eyes were placed in one of 4 wells of a well culture plate containing 250 μ l of minimal essential medium (MEM, Gibco), 10 μ Ci ³H-leucine (specific activity 110 Ci mmol⁻¹) and 0.5 μ l of ascites fluid from JONES hybridoma tumours. The retinal quadrants were incubated in this medium for 6 h at 37 °C, washed thoroughly in MEM and incubated for 1 h with ¹²⁵I-labelled F(ab)₂ fragments of an affinity-purified rabbit anti-mouse IgG directed against both heavy and light chains. The tissue was washed and processed for γ and β counting. ¹²⁵I counts were subsequently normalized to the amount of ³H-leucine taken up and incorporated in each quadrant to control variations in the tissue volume in each well.

fetal rodent eye^{27,28}. Thus the change from a symmetrical to a graded distribution of the JONES antigen between E13 and E17 could reflect a bulk tissue movement, continued expression by the earliest differentiating regions and a gradual spread of expression to ventral retina as the component cells reach the critical developmental stage. But this is unlikely to account for the continued expression of the gradient as, during the eight day period E17-P3 when the gradient of JONES binding is pronounced, even the youngest post-mitotic cells in the ventral regions of E17 retina attain the post-mitotic age and differentiate beyond the stage of dorsal retinal cells at E17 when the dorso-ventral gradient is first apparent. Therefore the differential expression of the JONES antigen is related to retinal position rather than to the post-mitotic age of individual retinal cells or to the differentiated state of a retinal region.

Molecules involved in establishing visual system topography are thought to be expressed in a graded pattern across the population of retinal ganglion cells so that they bias the selectivity of optic tract axons for different subregions of their central terminal fields²⁹. However there is relatively little information on these molecules. The expression of JONES antigen in retina

among cells from dorsal or ventral regions of chick retina¹⁰. Topographically appropriate binding of retinal cells to tectum was also observed¹¹, in experiments providing evidence for a double gradient where a protease-sensitive molecule controls the preferential adhesion of ventral retinal cells to dorsal tectum and a protease-insensitive molecule mediates the preferential adhesion of dorsal retinal cells to ventral tecta. The selectivity of binding of dorsal retinal cells was abolished by treatment with B-N-acetylhexosaminidase and could be mimicked by lecithin vesicles containing G_{M2} ganglioside¹². A study using high performance liquid chromatography on lipid extracts of chick retina has recently demonstrated slightly higher endogenous levels of G_{M2} in ventral retinal quadrants than in dorsal retinal quadrants¹³. The JONES antibody does not react with chick tissue. However, the initial biochemical characterization of the JONES antigen presented in this paper and studies using enzymatic treatments of extracts¹⁴ suggest that it too may be a ganglioside. Monoclonal antibodies to ganglioside antigens (for example, 18B8^{15,16}, AbR24^{17,18} and D1.1¹⁹⁻²¹) have been shown to vary in their cytoarchitectonic distribution with age in developing nervous system but no differences in binding based solely on topographic position have been described. To date, only one other molecule, TOP, has been found with a pronounced topographically varied distribution across the retina²². In binding assays the TOP molecule was nearly 12 times more concentrated in dorsal than ventral E14 chick retina²³. However, it has not been possible to demonstrate a gradient of anti-TOP binding with immunocytochemistry. The JONES antigen is unlikely to be related to TOP since the TOP molecule appears to be glycoprotein²³.

JONES binding was observed in regions of the retinal epithelium shortly after the genesis of the first post-mitotic cells. In the early fetus this region is in the centre of the eye. In the chick²⁴ and frog eye^{25,26} the first regions of the retina to have post-mitotic cells become situated in dorsal retinal quadrants because of the later addition of cells to ventral retina. Several observations suggest that similar displacements occur in the



Fig. 4 Biochemical characterization of the JONES antigen using TLC/immunoblot analysis. *a*, The JONES antigen partitions into the aqueous phase of a Folch extract and is detected as a single band on immunoblots of lipids resolved by TLC. This band migrates to a position between that of G_{M2} and G_{M1} ganglioside standards run at the same time. (1) whole lipid extract; (2) organic phase; (3) aqueous phase; (4) G_{D1a} ; (5) G_{M1} ; (6) G_{M2} . 1–3, immunostaining of the nitrocellulose blot of a TLC plate; 4–6, resorcinol staining of purified standards run at the same time. *b*, The epitope recognized by JONES antibody contains sialic acid as it is removed by treatment with neuraminidase (+) but not buffer (–).

Methods. P4 rat retinas were homogenized in chloroform/methanol (2:1) to give a crude lipid extract³⁶. This was partitioned into organic and aqueous phases by 0.1 M KCl⁹. Gangliosides and other highly polar lipids (including some phospholipids) partition into the aqueous phase, whereas most neutral glycolipids and some less polar gangliosides partition into the organic phase. The sialic acid content of the fractions was determined by the resorcinol method³⁷. Samples containing 0.3–0.4 μ g sialic acid were applied to a precoated HPTLC plate (Merck, silica gel 60, 0.2 mm thick) and developed with chloroform/methanol/0.25% aqueous $CaCl_2$ (60:40:10). Chromatograms were dried, wetted with isopropanol/water (2:1) and blotted onto nitrocellulose³⁸. The nitrocellulose blot was blocked in 5% normal goat serum and incubated with JONES antibody for 4 h. After washing, the blot was incubated in peroxidase-conjugated goat anti-mouse IgG (H + L) for 2–4 h and washed. Bound antibody was visualized by incubation with 3,3' diaminobenzidine. Ganglioside standards were run on parallel lanes as controls and were visualized by resorcinol staining³⁷. For neuraminidase digestion the aqueous phase retinal lipids were dried under N_2 and resuspended in 0.1 M sodium acetate, pH 5.5, with or without neuraminidase (Sigma type X from *Clostridium perfringens*) at 8 mU per μ g sialic acid. Incubations were carried out for 15 h at 37 °C. Digested lipids were extracted into chloroform:methanol, 2:1, dried under N_2 and resuspended in a small volume of chloroform:methanol, 2:1. Samples were run and analysed by TLC/immunoblot as described above.

coincides with the formation of central retinal connections. The first appearance of the JONES antigen in the retina on E12–13 is at the start of the period when the retinal ganglion cell axons first leave the eye³⁰. These axons begin to terminate in the superior colliculus on E17 when the JONES gradient first becomes apparent in the retina. Throughout the period when the retinotectal map is laid down, the JONES antigen maintains its graded distribution in the cellular layers of retina and can also be localized to the retinal regions of optic fibre growth. Moreover, recent evidence indicates that the JONES antigen is also present in all the diencephalic and midbrain regions invaded by the retinal axons^{31,32}.

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Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule

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The neural cell adhesion molecule (N-CAM) has been implicated in morphogenetic events during formation of the nervous system^{1,2}. Three forms of N-CAM exist, all glycoprotein chains, of relative molecular masses 180,000 (180K), 140K and 120K (N-CAM₁₈₀, N-CAM₁₄₀ and N-CAM₁₂₀) which are differentially expressed on neural cell types and during development^{3–5}. The three chains are thought to carry similar if not identical amino-acid sequences on their extracellular amino-terminal domains, but differ in the length of their carboxy-terminal cytoplasmic region^{6–8}. They occur in highly sialylated embryonic and less sialylated adult forms^{9,10}. N-CAM₁₈₀ is selectively expressed in more differentiated neural cells and may play a role in the stabilization of cell contacts⁵. To investigate this, we have studied in the surface membrane of a mouse neuroblastoma cell line N2A the lateral mobility of the two predominant forms of N-CAM, N-CAM₁₈₀ and N-CAM₁₄₀, as a function of differentiation. Here we report that as judged by fringe pattern photobleaching, the surface mobility of N-CAM₁₄₀ is higher than that of N-CAM₁₈₀, suggesting an association of N-CAM₁₈₀ with the cytoskeleton or other stabilizing factors. We also show that brain spectrin, a membrane-cytoskeleton linker

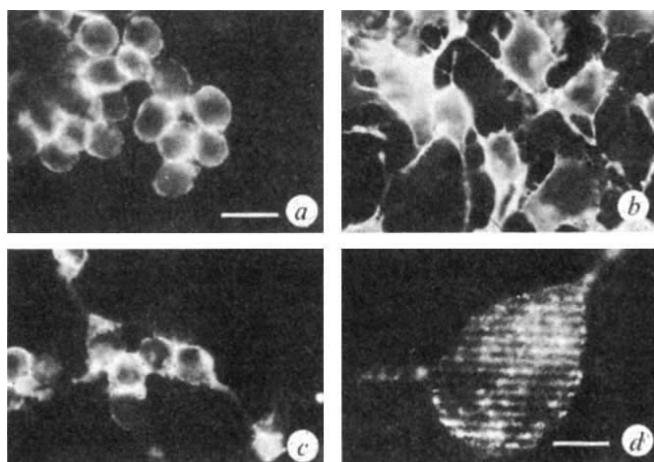


Fig. 1 Immunofluorescence localization of N-CAM in monolayer cultures of the mouse C1300 neuroblastoma clone N2A (ref. 35). Cells were stained with Fab fragments³⁶ of monoclonal antibody to mouse N-CAM (clone H28.123)^{37,38} coupled to rhodamine (lissamine rhodamine B sulphonylchloride, Kodak)³⁹. *a*, N2A cells maintained on coverslips coated with poly-L-lysine for 1 day in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). *b*, N2A cells maintained for 1 day *in vitro* on coverslip coated with laminin (5 μg per ml DMEM for 12 h at 4°C) in DMEM supplemented with 0.2% FCS. *c*, N2A cells cultured on coverslip coated with laminin for 7 days *in vitro* in DMEM supplemented with 0.2% FCS. *d*, Cells maintained as in *c* and illuminated with a fringe pattern. Scale bars, 30 μm (*a*, *b*, *c*) and 10 μm (*d*).

Methods. Cells were maintained under exponential growth conditions in plastic tissue culture flasks and subconfluent cells were trypsinized in the presence of EDTA as described²⁴, before being seeded at a density of 1×10^5 cells per ml on round 18 mm diameter glass coverslips under the culture conditions stated above. Live cells were incubated for 20 min at 0°C with rhodamine-labelled Fab fragments of monoclonal N-CAM antibody (30 μg ml⁻¹) in Dulbecco's phosphate buffered saline (pH 7.3). They were rinsed 4 times with ice cold Hank's balanced salt solution, pH 7.3, supplemented with 0.1% bovine serum albumin. The coverslips were then subjected to fluorescence microscopy (*a-c*) after fixation with paraformaldehyde⁴⁰ or to photobleaching (*d*). For photobleaching, they were placed in a circular Plexiglass chamber containing 400 μl of the same medium. The chamber was quickly sealed on both faces with molten paraffin and transferred to the cold stage of the photobleaching microscope^{13,14}. The cultures were maintained at 4°C and a field diameter of 28 μm was illuminated by two coherent laser beams that create a fringe pattern with an interfringe spacing of 1.85 μm.

protein¹¹, binds only to N-CAM₁₈₀. The immobilization of N-CAM in differentiated N2A cells is achieved by a shift in expression from N-CAM₁₄₀ to N-CAM₁₈₀.

The mouse neuroblastoma cell line N2A can be maintained, depending on the culture conditions, in proliferating, morphologically undifferentiated and stationary (morphologically differentiated) states¹². Under these conditions N2A cells only express the adult form of N-CAM. When cultured in low serum conditions or on laminin-coated coverslips, the cells stop proliferating and within one day of plating change from round cells with no processes to become more flattened, with numerous, sometimes branched neurites with growth cones, lamellipodia and filopodia (Fig. 1*b*). During the next 6–8 days, neurites become longer and less numerous (Fig. 1*c*). In low serum and with dimethyl sulphoxide, cell bodies assume a less flattened shape, but stop proliferating and extend neurites as on laminin. Because proliferation ceases under both conditions, differentiation does not alter the extent of cell contact except for the relatively small addition of the contacts made by outgrowing neurites. When laminin is used as substrate, N2A cells show a

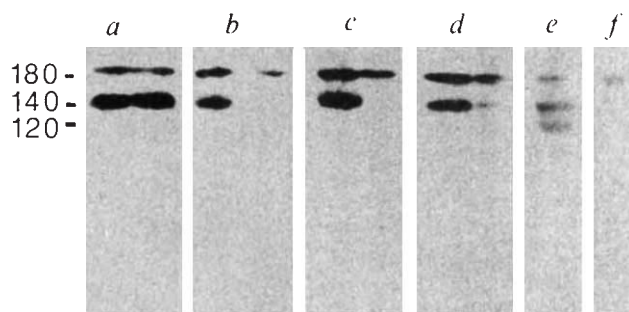


Fig. 2 Western blot analysis of: *a-d*, N-CAM expression by N2A cells maintained *in vitro* for 1 and 4 days (left and right lanes, respectively); *e*, immunoaffinity purified N-CAM; and *f*, radio-labelled brain spectrin bound to N-CAM. *a*, Cells seeded on poly-L-lysine-coated plastic flasks in DMEM supplemented with 10% FCS. *b*, Cells seeded on laminin-coated plastic flasks (see Fig. 1) in DMEM supplemented with 0.2% FCS. *c*, Cells plated on poly-L-lysine-coated plastic flasks in DMEM supplemented with 0.2% FCS and 2% dimethyl sulphoxide. *d*, Cells seeded on poly-L-lysine-coated plastic flasks in DMEM supplemented with 0.2% FCS. N2A cells were homogenized and further dissociated by ultrasonification (5 × 3 s). NP40 was then added to a final concentration of 0.5%. The homogenate was stirred for 30 min on ice, clarified by centrifugation for 3 min at 200g (Biofuge) and immediately boiled in sample buffer, separated by SDS-PAGE (7% slab gels), transferred to nitrocellulose filters, incubated with polyclonal antibodies to mouse N-CAM followed by incubation with ¹²⁵I-labelled protein A (2.5 μg ml⁻¹, 30 mCi mg⁻¹; Amersham) and autoradiographed²⁵. *e*, Affinity-purified N-CAM from adult mouse brain⁵ separated by SDS-PAGE was transferred to nitrocellulose filters, incubated with polyclonal rabbit antibodies to mouse N-CAM then horseradish peroxidase-coupled anti-rabbit Ig-antibody (Cappel) and developed with chloronaphthol as described²⁵. *f*, Incubation of affinity-purified N-CAM (as in *e*) with ¹²⁵I-labelled brain spectrin⁴¹ following the instructions of the supplier (Amersham) in a solid phase radioligand assay⁴². ¹²⁵I-labelled brain spectrin (specific activity 340,000 c.p.m. per μg) was used at a concentration of 1 μg ml⁻¹ in 10 mM phosphate buffer, pH 7.4, containing 4% bovine serum albumin. Apparent relative molecular masses (× 10⁻³) are indicated on the left.

morphologically differentiated phenotype within a day (Fig. 1*b*), but express the two N-CAM components as in undifferentiated cells. It takes four more days for the gradual loss of N-CAM₁₄₀ (Fig. 2). When N2A cells are cultured on poly-L-lysine in dimethyl sulphoxide, the disappearance of N-CAM₁₄₀ correlates with morphological differentiation⁵ (Fig. 2). We define undifferentiated cells as those that express both N-CAM₁₈₀ and N-CAM₁₄₀ and differentiated ones as those that only express N-CAM₁₈₀.

Lateral mobilities of cell surface constituents can be studied by determining the rate of diffusion of the fluorescence-labelled molecules from unphotobleached into photobleached areas. We created a series of stripes alternatively bleached and not bleached by using fringe pattern illumination (Fig. 1*d*). The decay in contrast of the periodic fluorescence concentration profile was measured as a function of time after pattern photobleaching^{13,14}. To study the lateral mobility of N-CAM components, they were labelled using Fab fragments of a monoclonal antibody against N-CAM, directly conjugated with rhodamine. This antibody recognizes both forms of N-CAM^{15,16}, does not interfere with adhesion¹⁶ and did not dissociate cell-cell or cell-substrate contacts in our experiments. As a control, we used rhodamine-labelled Fab fragments from polyclonal antibodies directed against mouse liver membranes. The antibodies react with an unrelated 130K antigen in undifferentiated and differentiated neuroblastoma cells (not shown). Single measurements were limited to a spot of 28 μm diameter, so that one cell and parts of neighbouring cells are photobleached. Data therefore represent the average N-CAM mobility in the surface membranes with and without cellular contact. This contact does not

Table 1 Lateral mobility of N-CAM and 130K antigen on surfaces of cultured neuroblastoma cells

Conditions of cell culture	a	b	c	d
Days in culture	1-3	1	7-9	7-9
DMEM supplemented with	10% FCS	0.2% FCS	0.2% FCS	0.2% FCS + 2% DMSO
Coverslips coated with laminin	—	+	+	—
Morphological differentiation*	—	+	+	+
Expression of N-CAM components (kilodaltons)	180, 140	180, 140	180	180
Lateral mobility of N-CAM				
Mobile fraction [†] (%)	87 ± 2	87 ± 2	24 ± 3	17 ± 1
Diffusion coefficient [†] ($\times 10^{11}$ cm ² s ⁻¹)	11.1 ± 1.1	14.7 ± 1.5	15.4 ± 2.4	10.3 ± 1.3
Number of determinations	26	26	10	23
Lateral mobility of 130K antigen				
Mobile fraction [†] (%)	91 ± 3	90 ± 2	83 ± 3	25 ± 1
Diffusion coefficient [†] ($\times 10^{11}$ cm ² s ⁻¹)	8.9 ± 1.1	9.4 ± 1.5	13.1 ± 1.6	10.0 ± 1.4
Number of determinations	9	11	11	16

* Criteria for morphological differentiation are the following: undifferentiated, cells with round cell bodies and without processes; differentiated, cells with flattened cell bodies and several processes, sometimes branched, with growth cones and filopodia. DMSO, dimethyl sulphoxide.

[†] The fraction of mobile fluorochrome-coupled Fab fragments bound to live N2A cells was estimated from the modulated emission of fluorescence (MEF) curves (see Fig. 3) by fitting the data to a sum of two exponential components plus a constant baseline using a program developed by Vogel³⁴. The decaying traces of lateral diffusion were found to contain essentially two terms: a rapid diffusion component in the range of 10^{-10} cm² s⁻¹, referred to here as the mobile fraction and a second component, either resulting from a very small diffusion coefficient, ($D < 4 \times 10^{-12}$ cm² s⁻¹) or due to subtle changes in the shape of the cell or even to a partial dissociation of Fab fragments. The diffusion coefficient reported here is given by $D = l^2/(4\pi^2 T)$, where l is the interfringe spacing (1.85 μ m) and T the exponential decay time constant of the mobile fraction. The data are expressed as the mean $\pm$ s.d.

appear to be significant in determining the membrane mobility of N-CAM. Photobleaching measurements were performed at 4 °C to avoid internalization of antigen-antibody complexes, which was considerable with the 130K antigen and least significant with N-CAM₁₄₀.

After pattern photobleaching, the randomization of the immunolabelled N-CAMs occurred with two distinct diffusion kinetics (Fig. 3). In undifferentiated cells, the decay of the periodic fluorescence concentration profile is rapid (Table 1) and goes virtually to completion (Fig. 3a, curves A, B). Here, most of the N-CAM is of the N-CAM₁₄₀ form (see Fig. 2a, b) that has a high lateral mobility. In differentiated cells, there is a slow and incomplete randomization of N-CAM, characterized by a persistent periodic contrast (Fig. 3a, curve C). This immobile fraction of N-CAM is primarily N-CAM₁₈₀ which is exclusively present in differentiated cells (Fig. 2c). In contrast, the randomization of the 130K surface antigen is nearly complete during the time course of the experiment (Fig. 3b, curves A, B, C). The mobile fraction and respective mean diffusion coefficients were calculated (Table 1). N-CAM shows a highly mobile fraction which amounts to ~87% of all immunolabelled N-CAM molecules in the undifferentiated state and ~24% in the differentiated state of N2A cells. The diffusion coefficient is 1.1×10^{-10} cm² s⁻¹ at 4 °C for undifferentiated cells, and approximately the same for differentiated cells. The 130K component has a mobile fraction amounting to approximately 91% of all immunolabelled 130K molecules in the undifferentiated state, 83% for cells induced to differentiate on laminin, and 25% on cells induced to differentiate in low serum and dimethyl sulphoxide for 7-9 days. Thus, the 130K component has a similar mobile fraction in undifferentiated and differentiated cells provided that differentiation is induced under the more physiological conditions of laminin as substrate. We cannot explain why cells allowed to differentiate in the presence of dimethyl sulphoxide contain a smaller fraction of mobile components. More importantly, however, the mean diffusion coefficient of the 130K component is nearly the same (8.9 – 13.1×10^{-11} cm² s⁻¹) in both the undifferentiated and differentiated states and does not depend on whether cells are induced to differentiate by dimethyl sulphoxide or by laminin. These experiments show a close correlation between the ratio of expression of N-CAM₁₄₀ to N-CAM₁₈₀ and the ratio of mobile to immobile N-CAM fractions in undifferentiated and differentiated states.

To test whether N-CAM₁₈₀ is linked to cytoskeletal components, immunoaffinity-purified N-CAM from adult mouse brain was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and either stained with antibodies to N-CAM (Fig. 2e) or incubated with radio-labelled brain spectrin (Fig. 2f). Brain spectrin¹¹ binds exclusively to N-CAM₁₈₀.

We have shown that differentiation of neuroblastoma cells is accompanied by increased expression of N-CAM₁₈₀ which is less mobile in the surface membrane than N-CAM₁₄₀, the form expressed in undifferentiated cells. The rate of diffusion of N-CAM₁₄₀ is relatively high and similar to that of highly mobile cell surface glycoproteins (see, for instance, refs 17, 18). The immobilization of N-CAM is manifested by a decrease in the mobile N-CAM fraction (the N-CAM₁₄₀ component). This reduction in mobility is not due to a general differentiation-induced immobilization because another, unrelated membrane component of 130K has the same mobility in undifferentiated and differentiated cells. Morphological differentiation *per se* does not correlate with restriction in surface mobility or disappearance of N-CAM₁₄₀: when plated on the extracellular matrix component laminin, a physiological substrate for neurones¹⁹⁻²¹, morphological differentiation of neuroblastoma cells is induced within one day. At this stage, both N-CAM₁₄₀ and N-CAM₁₈₀ are expressed and have a surface mobility characteristic of morphologically undifferentiated neuroblastoma cells. Morphological differentiation thus precedes N-CAM₁₈₀ immobilization. Failure to detect differences in surface mobilities between N-CAM and other cell surface molecules on neural cells²² may have been due to an inability to distinguish between the individual N-CAM components.

The immobilization of N-CAM₁₈₀ in differentiated N2A cells could be related to an interaction with other cell surface components or with the cytoskeleton. We have previously shown that N-CAM₁₈₀ first appears in the central nervous system on post-mitotic neurones, whereas N-CAM₁₄₀ and N-CAM₁₂₀ appear at earlier developmental stages^{5,23}. The appearance of N-CAM₁₈₀ is accompanied by the expression of another cell adhesion molecule, the L1 antigen²⁴⁻²⁶, before the first migration of granule cell neurones in the cerebellar cortex²⁷. N-CAM and L1 act synergistically in reaggregating cerebellar cells taken at the time of early postnatal granule cell migration²⁵. L1 is associated with N-CAM₁₈₀, but not with N-CAM₁₄₀, as shown by

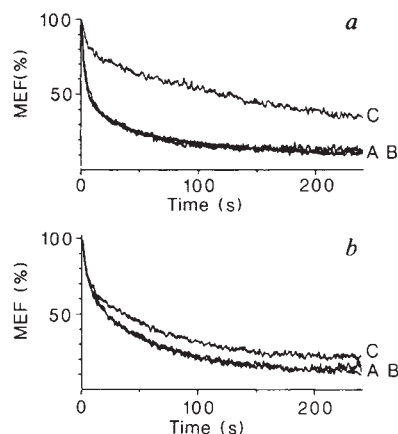


Fig. 3 Modulated recovery of fluorescence after fringe pattern photobleaching of N2A cells. *a*, Rhodamine-labelled Fab fragments of monoclonal N-CAM antibodies; *b*, rhodamine-labelled Fab fragments of polyclonal antibodies to a 130K antigen; A, N2A cells plated on poly-L-lysine in DMEM supplemented with 10% FCS and maintained in culture for 1–3 days; B, N2A cells plated on laminin and maintained in DMEM supplemented with 0.2% FCS for 1 day in culture; C, N2A cells plated on laminin and maintained in DMEM supplemented with 0.2% FCS for 7–9 days *in vitro*.

Methods. Polyclonal antibodies (raised in rabbits) against mouse liver membranes²⁷ recognize a single band of 130K relative molecular mass in Western blots of NP40 extracts of N2A cells (unpublished results). N2A cells were maintained on glass coverslips, labelled with fluorescent antibody and illuminated with a fringe pattern as described in Fig. 1. Measurements were done on individual cells with or without contact sites. During the bleaching pulse of 200 ms ($1,500 \text{ W cm}^{-2}$, $\lambda = 514 \text{ nm}$), between 10–20% of the total rhodamine-labelled Fab fragments present on the illuminated fringes with an interfringe spacing of $1.85 \mu\text{m}$ (see Fig. 1*d*) are irreversibly bleached at the origin of the time axis. This low level of bleaching was used to minimize photodamage to the cells. After pattern photobleaching, the decay of the periodic fluorescence concentration profile was monitored continuously by an interrogation fringe pattern (less than 1 W cm^{-2}) that scans back and forth (3 kHz)^{13,14}. The resulting modulated emission of fluorescence (MEF) was detected using a photomultiplier coupled to a lock-in amplifier, and stored after digitalization with a time resolution of 240 ms. Only the periodic distribution of bleached versus non-bleached fluorophores contributes to the signal. The amplitude of MEF which is a measure of the periodic contrast created by the fringe pattern photobleaching was normalized with respect to its peak value (100%). The MEF decayed as a function of time because of the randomization of fluorochrome-labelled antibodies. Each trace represents the average value of several measurements (n): $n = 26$ (*a*, A and B), $n = 10$ (*a*, C), $n = 9$ (*b*, A) and $n = 11$ (*b*, B and C) (see Table 1). Per coverslip, 5–10 individual photobleaching measurements could be performed within 1 h of mounting. The standard deviations of the population curves were smaller than the peak-to-peak level of the noise of the averaged curve.

antibody-induced co-redistribution on the surface of neuroblastoma cells²⁸. Both L1 and N-CAM₁₈₀ are concentrated at contact points between neighbouring neuroblastoma cells, whereas N-CAM₁₄₀ shows a more uniform distribution in both normal and transformed neural cells^{5,29}. The membrane-cytoskeleton linker protein brain spectrin (for reviews, see refs 30, 31) is also more concentrated at these contact points and copurifies with N-CAM₁₈₀, but not with N-CAM₁₄₀ nor N-CAM₁₂₀ under the stringent conditions of immunoaffinity purification³². Combining these observations with the fact that N-CAM₁₈₀ shows a restricted lateral mobility in differentiated neuroblastoma cells suggests a direct cytoskeletal involvement in the restriction of lateral mobility, as in other systems^{17,30,31,33}. The membrane-cytoskeleton linker protein brain spectrin does

indeed bind exclusively to N-CAM₁₈₀. The association of N-CAM₁₈₀ with the cytoskeleton could be mediated by the longer cytoplasmic domain of N-CAM₁₈₀. Further stabilization of cell contacts could be achieved by conformational modifications in intracellular protein domains triggered by conformational changes on extracellularly domains and vice versa. It will be interesting to determine whether the restriction of N-CAM₁₈₀ within the surface membrane is induced by the preceding accumulation of the cytoskeleton at contact sites between neighbouring cells. Alternatively, N-CAM₁₈₀ could be induced to accumulate by L1 antigen or other components localized within the surface membrane of the same cell, or by an extracellular component(s). In any case, restriction of lateral mobility and accumulation at particular sites within the surface membrane would then lead to the stabilization of selective cell-cell contacts.

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Note added in proof: It has been recently reported^{43,44} that phosphatidylinositol is involved in the membrane attachment of NCAM₁₂₀. Therefore we would expect the surface mobility of NCAM₁₂₀ to be identical or higher than the one reported here for NCAM₁₄₀.

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Endfeet of retinal glial cells have higher densities of ion channels that mediate K^+ buffering

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A major function of glial cells in the central nervous system is to buffer the extracellular potassium concentration, $[K^+]_o$. A local rise in $[K^+]_o$ causes potassium ions to enter glial cells, which have membranes that are highly permeable to K^+ ; potassium then leaves the glial cells at other locations where $[K^+]_o$ has not risen. We report here the first study of the individual ion channels mediating potassium buffering by glial cells. The patch-clamp technique was employed to record single channel currents in Müller cells, the radial glia of the vertebrate retina. These cells have 94% of their potassium conductance in an endfoot apposed to the vitreous humour¹, causing K^+ released from active retinal neurones to be buffered preferentially to the vitreous^{2,3}. Recordings from patches of inward-rectifying K^+ channel mediates potassium buffering at both cell locations. The non-uniform density of K^+ conductance is due to a non-uniform distribution of one type of K^+ channel, rather than to the cell expressing high conductance channels at the endfoot and low conductance channels elsewhere on the cell.

Cell-attached patch recordings⁴ were made from the cell body or terminal endfoot membrane of Müller cells isolated from

axolotl retinæ (Fig. 1a). As a rule, cell body recordings revealed no channels or only one channel, while recordings from the terminal endfoot membrane showed five or more (up to 50) channels. Occasionally, however, cell body patches contained up to four channels and endfoot patches sometimes showed less than five channels.

Over the voltage range where single channel transitions were seen, at potentials hyperpolarized from the channel reversal potential, the open channel current-voltage relations were linear with a conductance that was higher when the pipette $[K^+]_o$ was higher (Fig. 1b). The current-voltage relations of membrane patches (Fig. 2) suggest that the channels exhibit inward rectification, that is, the channel conductance is much smaller at potentials more positive than the reversal potential than at potentials below it. This inward rectification may reflect the properties of the open channel I-V relation, or the presence of voltage-dependent gating, too fast to be detected as discrete channel jumps, decreasing the channel open probability at depolarized potentials^{5,6}. Extrapolated reversal potentials for the channels change by approximately 52 mV for a ten-fold change in $[K^+]_o$, indicating that the channels are largely potassium-selective (Fig. 3a). The conductance is approximately proportional to the square root of $[K^+]_o$ (Fig. 3b), as found for inward rectifier channels in other preparations^{5,7,8}. The potassium-selectivity and the $[K^+]_o$ -dependence of the single channel conductance are indistinguishable for endfoot and cell body channels.

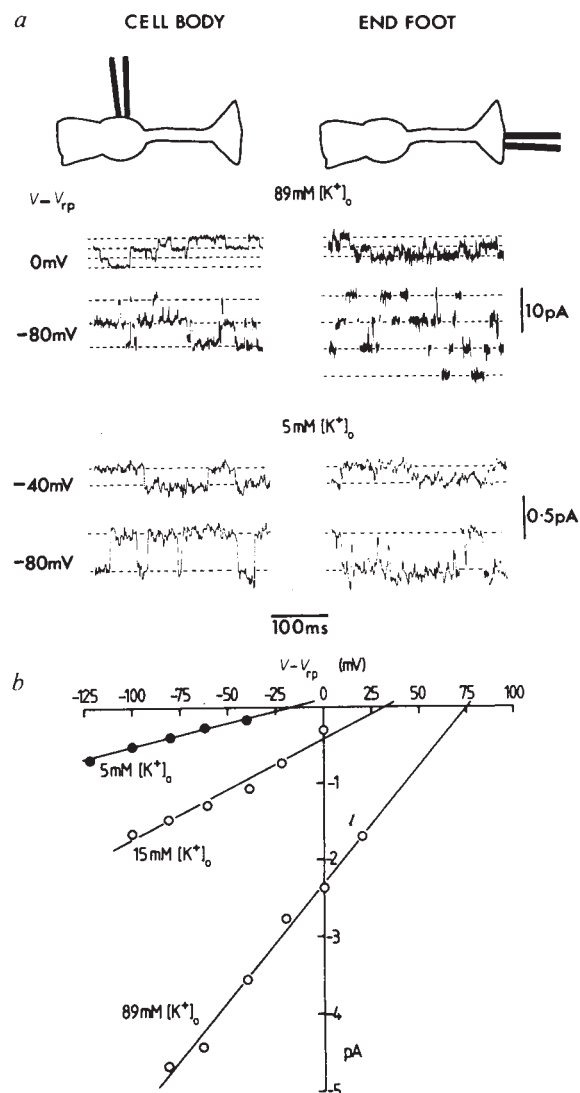


Fig. 1 *a*, Cell-attached recordings and *b*, single channel current-voltage plots for the cell body or terminal endfoot membrane of Müller cells isolated from enzymatically-dissociated¹ retinæ of the axolotl (*Ambystoma mexicanum*). *a*, Records are shown from four different patches, two studied at each cell location, with 5 mM or 89 mM K^+ solution in the patch pipette (1.5 mM K^+ solution bathing the cell). Note the different current scales for the two $[K^+]_o$ values. Inward current shown downwards. For each patch, data are shown at the two potentials shown on the left relative to V_{rp} , the resting potential ($V - V_{rp}$ is minus the command potential applied to the pipette). Conventional micro-electrode recordings from 93 isolated Müller cells gave a mean resting potential of $V_{rp} = -88$ mV (± 7 mV s.d.). Channel opening is downward (increased inward current). Dashed lines at constant current intervals show the single channel current levels. A patch containing 3 channels has been chosen to display the 89 mM $[K^+]_o$ cell body data; typically there were one or no channels in cell body patches. The K^+ channels characterized here were by far the most common channels observed. They contributed the majority (typically 85% in 50 or 89 mM $[K^+]_o$) of the conductance in membrane patches studied at both the cell body and the endfoot: the remaining conductance (not showing single channel transitions) was probably the conductance of the seal between the patch electrode and the cell, since its reversal potential was always around a pipette potential of zero rather than at the potassium reversal potential.

Cells were maintained at 20–25 °C in perfusate containing (mM): NaCl 90.5; KCl 1.5; $MgCl_2$ 0.5; $CaCl_2$ 3; glucose 15; hydroxyethyl piperazine ethane sulphonic acid (HEPES) 20; adjusted to pH 7.3 with NaOH; bubbled with O_2 . Solutions in the patch pipette were as above but with KCl replacing NaCl to give any desired $[K^+]_o$ value (5, 15, 30, 50 or 89 mM). *b*, Current-voltage plots obtained from records like those in *a*, for three patches studied with different concentrations of K^+ in the patch pipette. Data for 5 mM $[K^+]_o$ are from an endfoot; 15 and 89 mM $[K^+]_o$ data are from cell body patches. Each point plotted is the average of 10–20 single channel transitions. Abscissa gives membrane potential relative to the resting potential in 1.5 mM K^+ solution (–88 mV).

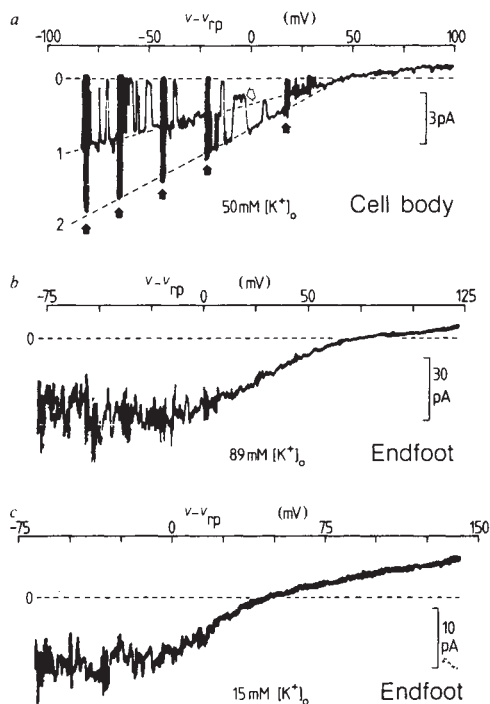
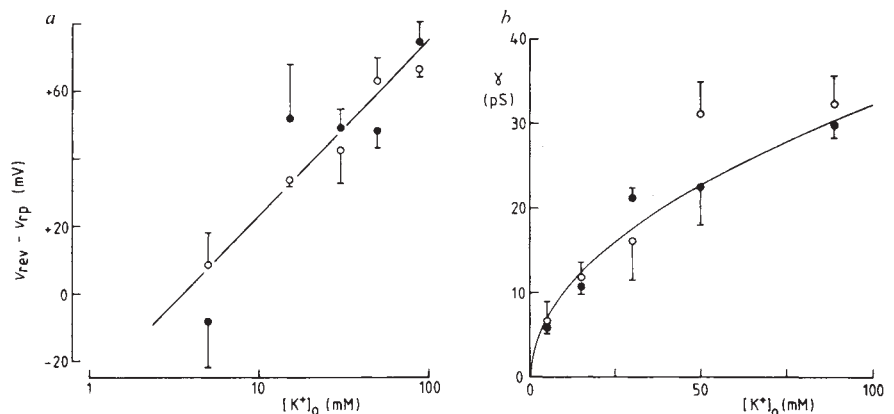


Fig. 2 Inward rectification of the Müller cell potassium channels. *a*, Current-voltage relation of a cell body membrane patch containing 2 channels, with 50 mM K^+ in the patch pipette. *b, c*, Current-voltage relations of endfoot patches showing inward rectification with 89 mM (*b*) or 15 mM (*c*) K^+ in the pipette. *a*, Current through the seal conductance (approximately 30% of the conductance of one open channel at negative potentials, with a reversal potential of 0 mV) was subtracted electronically from this record. The membrane potential (abscissa, given with respect to the resting potential of -88 mV) was ramped at roughly 70 mV s^{-1} from $V - V_{rp} = +100$ to -100 mV. Dashed lines connect values when no channels are open (0), when only one is open (1) and when both are open (2). At the voltages labelled with filled arrows, the voltage ramp was stopped to allow time for transitions to occur between all three states (0, 1 and 2). For $V - V_{rp} < -25$ mV, only 1 or 0 channels are open most of the time because of the low channel open probability, while for $V - V_{rp} > -25$ mV both channels are often open because the open probability is higher (see Fig. 4). For $V - V_{rp} > +50$ mV, above the channel reversal potential where the probability of channel opening is high (Fig. 4), the outward current carried by the channels is much less than would be predicted by extrapolating the channel I-V relation from more negative potentials. At one point, just negative to 0 mV, one channel enters a conductance substate (open arrow). Such substates were seen both at the endfoot and the cell body. At the cell body they were seen for 20% of channels and for these channels the probability of being in a substate was about 0.1 at -90 mV. *b, c*, Endfoot patches containing 24 and 30 channels respectively (calculated by dividing the inward current at $V - V_{rp} = 0$ mV by the open probability at that potential (0.64, see Fig. 4) and by the single channel current). Flattening of the current for hyperpolarization below $V - V_{rp} = 0$ mV reflects the voltage-dependence of the channel open probability (Fig. 4). For example, multiplying the curve describing the voltage-dependence of P_{open} in Fig. 4 by an ohmic I-V relation with a reversal potential of $V - V_{rp} = 70$ mV predicts that the maximum inward current through the channels should be near $V - V_{rp} = -30$ mV and that the current at $V - V_{rp} = -80$ mV will be 84% of this maximum value, in reasonable agreement with the data in *b*. Above the reversal potential the outward current is much smaller than predicted by extrapolating from more negative potentials. No subtraction of the seal conductance was applied to records *b* and *c*.

Fig. 3 *a*, Reversal potentials (measured relative to the resting potential of -88 mV) extrapolated from I-V plots like those in Fig. 1*b* for $\circ$, cell body and $\bullet$, endfoot patches studied with 5 different pipette $[K^+]_o$ solutions. Each point is the average of 1-7 values; bars show standard errors. The line through the points is a least squares fit, with a slope of 52 mV per 10-fold change of $[K^+]_o$. *b*, Single channel conductance measured from I-V plots like those in Fig. 1*b*, for $\circ$, cell body and $\bullet$, endfoot patches studied with 5 different $[K^+]_o$ values. Curve has the form $\gamma = (A[K^+]_o)^{1/2}$ with $A = 10.24 (\text{pS})^2 (\text{mM})^{-1}$.

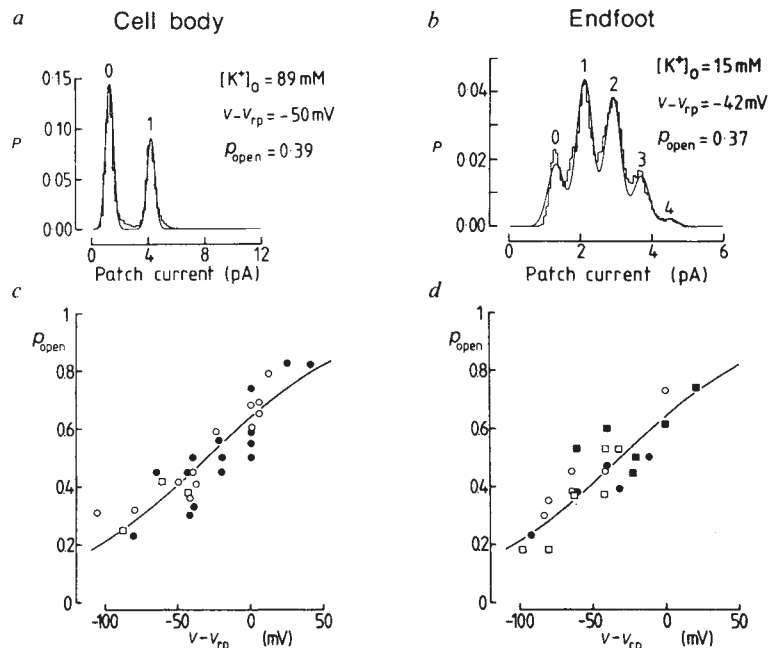


To investigate whether the probability of channels being open is higher at the endfoot than at the cell body, we constructed current amplitude histograms (Fig. 4*a, b*). Figure 4*c* and *d* shows the voltage-dependence of the steady-state open probability, calculated from such histograms, for endfoot and cell body channels. The open probability increases with depolarization, has similar values in endfoot and cell body channels and is independent of $[K^+]_o$ and $[Na^+]_o$.

We believe that the potassium channels characterized here are those responsible for mediating the spatial buffering of

potassium by Müller cells for the following reasons. First, the channels have the necessary potassium specificity for carrying out potassium buffering. Second, the dependence of single channel conductance on potassium concentration in Fig. 3 is similar to that found for the conductance of whole Müller cells (measured at -90 mV by whole-cell clamping, for $[K^+]_o$ up to 20 mM, data not shown). Lastly, although Müller cell bodies contain I_A -type and calcium-activated potassium currents, in addition to inward-rectifying potassium currents, the former currents are only activated by depolarization beyond

Fig. 4 *a, b*, Current amplitude histograms for determining the channel open probability, constructed from 10–40 second stretches of current at different potentials. *c, d*, The open probability of cell body (*c*) and endfoot (*d*) channels, obtained at different voltages from data like those in *a* and *b*, with different values of $[K^+]_o$: $\circ$, 89 mM K^+ ; $\bullet$, 50 mM K^+ ; $\blacksquare$, 30 mM K^+ ; $\square$, 15 mM K^+ . *a*, Cell body patch containing only one channel, held at a potential 50 mV hyperpolarized from the resting potential (at about -138 mV); 89 mM K^+ in the patch pipette. *P* is the probability per 165 fA bin. The two current levels (0 = no channels open, 1 = one channel open) are sufficiently separated on the amplitude histogram (by 2.95 pA) that the areas of their distribution peaks could be calculated directly. The smooth curve through each peak shows a gaussian distribution with a standard deviation of 0.28 pA. The open probability (area of 'open' peak/sum of areas of 2 peaks) was $P_{open} = 0.39$. *b*, An endfoot patch containing 4 channels, held 42 mV hyperpolarized from the resting potential (at about -130 mV), with 15 mM K^+ in the patch pipette. *P* is the probability per 66 fA bin. Curve through the points is a fit of the binomial prediction for the probability, $P(I)$, of a current amplitude, I , being seen $P(I) = \sum_{j=0}^N B(N, j) S(I - jI_1)$ where there are N channels present (4 in this case) each carrying an open channel current I_1 ; the current flowing when there are j channels open is jI_1 ; S is a gaussian function describing the noise present in the current (assumed, for simplicity to be independent of the number of channels open), $S(I) = \exp(-I^2/\sigma^2)$; and $B(N, j)$ is the probability of j of the N channels being open, obtained from the binomial prediction $B(N, j) = N! / [(N-j)!j!] P_{open}^j (1 - P_{open})^{N-j}$ where P_{open} is the open probability. For these data $P_{open} = 0.37$, $I_1 = 0.81$ pA and $\sigma = 0.22$ pA. *c, d*, Smooth curves through the points are best fits of the form $P_{open} = 1 / \{1 + \exp(-(V - V_{rp} - V_1)/k)\}$. The parameters V_1 and k were essentially identical at the cell body and endfoot: $V_1 = -30.5$ mV, $k = 53.2$ mV at the cell body, and $V_1 = -31.4$ mV, $k = 54.9$ mV at the endfoot. This voltage-dependence of the open probability appears to be due to true voltage-dependent gating, rather than a voltage-dependent block by external Na^+ ions as occurs for the inward rectifier in tunicate eggs⁸ and frog skeletal muscle⁹, since (1) the dependence of P_{open} on voltage is independent of the extracellular sodium concentration (the variations of $[K^+]_o$ were obtained by substituting NaCl for KCl in the patch pipette) and (2) replacing extracellular NaCl by choline chloride did not change the open probability.



-40 mV, which is probably more positive than physiological voltages¹⁰.

This is the first study of the individual ion channels responsible for potassium buffering by glial cells. It is also the first investigation attempting to account for the non-uniform distribution of K^+ conductance in glial cells in terms of the properties of individual ion channels. Newman¹⁰ suggested that potassium buffering might be mediated by different types of channels at the cell body and the endfoot. We have shown that there is just one type of channel present, with more channels being expressed at the endfoot than elsewhere. Recently, Newman¹¹ has shown that the non-uniform potassium conductance causing directed spatial buffering of potassium is not unique to Müller cells, but also occurs in optic nerve astrocytes. The mechanisms we have characterized in Müller cells may therefore be common to all glial cells carrying out potassium buffering.

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Cyclic GMP is involved in the excitation of invertebrate photoreceptors

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The hyperpolarizing receptor potential in vertebrate rod photoreceptors appears to be mediated by the second messenger, cyclic GMP. Injection of cGMP into rods^{1,2} or application of cGMP to inside-out membrane patches³ activates a conductance resembling that produced by light. Light produces a rapid reduction of cGMP in living rods⁴, leading to closure of sodium channels and membrane hyperpolarization. In most invertebrate photoreceptors the response to light is depolarizing. We have investigated whether cGMP is involved in controlling the increase in sodium conductance that underlies this depolarization⁵. We show here that injection of cGMP into *Limulus* photoreceptors produces a depolarization that mimics the receptor potential. We also show that the cGMP concentration of the squid retina increases rapidly during exposure to light. These results support the hypothesis that cGMP mediates the light-induced depolarization in invertebrate photoreceptors and suggests that vertebrate and invertebrate phototransduction may be more similar than previously thought.

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Fig. 1 Responses to injection of cGMP. Individual photoreceptor cells were mechanically isolated from a desheathed *Limulus* ventral nerve with a large suction pipette in order to distinguish the light-sensitive lobe. A microelectrode containing cGMP was placed in the R-lobe under the control of a motor-driven micromanipulator. Injections were monitored optically with an infrared television¹⁷. The microelectrode had 10 mM or 1 mM cGMP in stock solution of 300 mM K aspartate, 10 mM HEPES, pH 7.0. *a*, The response to a single photon (lower trace) compared with the response to a very small injection of cGMP (upper trace). *b, c*, Superimposed responses to pressure injection of increasing amounts of cGMP. Duration of injections is marked by bars below the voltage traces. The applied pressure was <1 p.s.i.

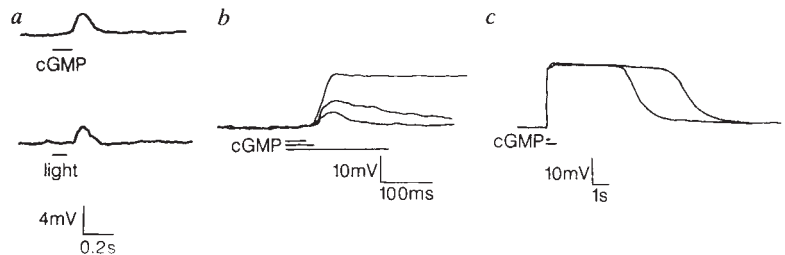
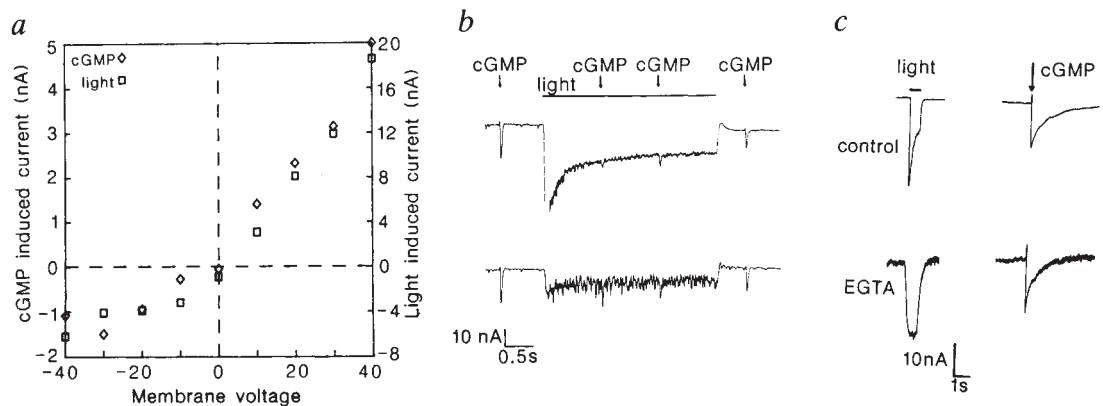


Fig. 2 Effect of voltage, background light, and intracellular Ca^{2+} buffer on cGMP-induced currents measured using two-electrode voltage-clamp. *a*, Current-voltage characteristics of light-induced and cGMP-induced currents. Light intensity $0.08 \mu\text{W cm}^{-2}$; currents are given in nA. *b*, Effect of adapting lights on the response to cGMP. A brief pulse of cGMP was injected at each arrow. Upper trace light intensity $0.05 \mu\text{W cm}^{-2}$, lower trace $0.005 \mu\text{W cm}^{-2}$. Note that the light-adapted cGMP response at the end of the upper trace recovered in the 30-s dark period between the upper and lower traces. *c*, Response to cGMP was not blocked by injection of EGTA from a second electrode. Note that this is a different cell and slower time base than in *b*. The responses on the left demonstrate that the light response after EGTA injection (lower left) did not display the decline from the initial transient phase to the plateau phase normally observed in the light response (upper left). This is evidence that EGTA buffered the change in intracellular calcium level that regulates light adaptation¹⁸. On the right are responses to cGMP injection in the dark (top right, before EGTA injection; bottom right, after EGTA injection). The reason for the increase in baseline noise after EGTA injection is unclear.



We pressure-injected cGMP into the cytoplasm of *Limulus* ventral photoreceptors. If the injection electrode was in a cGMP-sensitive region of the cell (see below), injections evoked a transient depolarization. For small injections, the response kinetics were similar to the kinetics of the light response (Fig. 1a). The size of the depolarization was smoothly graded with the strength of the injection (Fig. 1b); there was no indication that cGMP can evoke discrete waves⁶, as is characteristic of other agents that depolarize ventral photoreceptors⁷⁻⁹. With larger injections the response saturated; the only effect of further increases was to lengthen the response duration (Fig. 1c). Once the response began to fall, it fell with kinetics that were independent of the duration of the response. The saturation of the cGMP response was only 20–30 mV, much lower than the saturation of the light response (50–60 mV). This is as expected if the injected cGMP saturates a local subpopulation of channels. The cGMP would then be rapidly hydrolysed, but the voltage would not fall until the local cGMP concentration fell below saturation. The larger the injection, the longer cGMP levels would remain at saturation. Voltage-clamp measurements allowed further comparison of the responses to light and cGMP. cGMP induced a current with a reversal potential and voltage-dependence nearly identical to that of the light-induced current (Fig. 2a). The response to cGMP was reduced by an adapting light the same way as the response to a flash would be (Fig. 2b). Thus, in several respects the conductance activated by cGMP is similar to that activated by light.

Recently inositol trisphosphate (InsP_3) has been suggested as a messenger of excitation in invertebrate photoreceptors^{7,8}. One

difficulty with this hypothesis is that injection of calcium buffers blocks the excitation produced by InsP_3 (ref. 10) but enhances the maintained excitation produced by light¹⁸. Injection of calcium buffer (EGTA) did not block the response of a dark-adapted cell to cGMP (Fig. 2c). Thus both the response to light and cGMP can occur without a change in cytoplasmic calcium.

Not all regions of the *Limulus* ventral photoreceptor were sensitive to cGMP. The ventral photoreceptor is divided into lobes^{11,12}; the light-sensitive R-lobe (40 μm in diameter) contains rhodopsin in microvillar specializations of the plasma membrane. The A-lobe contains no microvilli. Microvilli are restricted to small subregions of the R-lobe. The sensitivity of the cell to cGMP was highly dependent on the position of the pipette. Injection of cGMP into the A-lobe was without effect. Injection of cGMP into the R-lobe often produced only small depolarizations (0–5 mV). By moving the injection electrode through the R-lobe, it was sometimes (<10%) possible to find a region in which injections produced large responses. The position dependence of the cGMP response was confirmed by making reversible movements in and out of the cGMP-sensitive region (Fig. 3a). Because of this position dependence, it was difficult to study the cGMP response. This probably explains the failure of previous efforts^{9,13} to detect a cGMP response.

Because of the low probability of obtaining responses to cGMP it was possible that these responses were artefacts. It was therefore important to see if other substances produced responses when injected into regions known to be responsive to cGMP. We used double-barrel θ micropipettes with one barrel containing cGMP, the other a test solution. Once a cGMP-

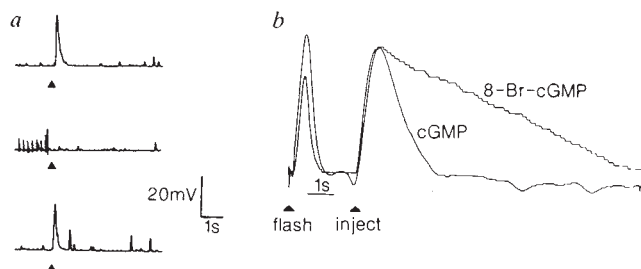


Fig. 3 Dependence of the response to cGMP on electrode placement and cGMP hydrolysis rate. *a*, Dependence of response to cGMP on electrode position. Upper trace, response to brief injection of cGMP (Δ); middle trace, after $\sim 10 \mu\text{m}$ movement of electrode (stepping motor caused series of artefacts) there was no response to cGMP injection; bottom trace, movement of micropipette back to original position restored the response. *b*, Response to the slowly hydrolysable analogue 8-Br-cGMP. A double-barrel pipette with 10 mM cGMP in one barrel and 10 mM 8-Br-cGMP in the other barrel was used. Injections given at arrows. Average responses from 7 injections of each are shown superimposed. Peak responses to 8-Br-cGMP and cGMP differed by $<20\%$, but are shown here normalized. Depolarizations at left are responses to test flashes.

sensitive region was found, larger amounts of the test solution were injected through the other barrel. We found that cGMP-sensitive regions showed no significant response to the injection stock, cAMP or 5'-GMP, but were sensitive to 8-Br-cGMP. The response to 8-Br-cGMP fell more slowly than the response to cGMP (Fig. 3*b*) as would be expected of this slowly hydrolysable analogue.^{14,15}

The very rapid hydrolysis of cGMP must limit its spread and may explain why the response to cGMP is more localized than the response to Ca^{2+} (ref. 21) or InsP_3 (refs 7, 8). We suspect that localization may also be due to restricted access to the microvillar membrane, possibly caused by the subhadromeric reticulum, a fenestrated internal membrane system that abuts the microvillar membrane¹⁰. Indeed, a quasi-independent compartment between the reticular and microvillar membranes might enhance transduction, since a change in the number of cGMP molecules leads to a concentration change inversely proportional to the volume over which cGMP is spread; a restricted diffusion therefore enhances the signal.

If cGMP is a messenger for excitation in invertebrate photoreceptors, cGMP levels must rise rapidly in light. We examined cGMP levels in squid (*Loligo paeili*) because its retina is large and primarily contains photoreceptors. Punches ($\frac{1}{4}$ inch diameter) of living retina and associated scleral material were obtained from dark-adapted animals under deep-red illumination and were frozen with a liquid nitrogen cooled hammer. The punch from one eye was frozen in the dark; that from the other eye was frozen 100–200 ms after the onset of illumination. cGMP was extracted by homogenizing the frozen retinas in cold HCl-ethanol (1:99), and measured by radioimmunoassay¹⁹. Dark levels of cGMP were 2.0 ± 0.3 (s.e.m., $n=23$) pmol per mg protein. Light levels were 1.7 ± 0.27 (s.e.m., $n=23$) times greater than dark levels, a significant increase ($t=1.87$, d.f. = 22, $P<0.05$, one tail). As this is the sum of cGMP changes over the whole punch of retina, the fractional change of cGMP in the transducing region is likely to be much larger than 1.7.

The finding of a light-induced increase in cGMP in the intact squid retina is consistent with the *in vitro* work of Saibil¹⁶ showing a light-induced rise of cGMP in homogenates of squid outer segments. Our general conclusion regarding the importance of cGMP is consistent with the early suggestion²⁰ that cyclic nucleotides are involved in invertebrate phototransduction. Previous work on vertebrate rods^{1–3} and the work reported here on invertebrate photoreceptors suggest that cGMP increases the sodium conductance in both types of cells. The

difference in sign of the receptor potentials appears to be due to a difference in sign of the light-induced change in cGMP. The decrease in cGMP concentration in vertebrates is due to activation of phosphodiesterase. Whether the rise in cGMP concentration in invertebrates is due to activation of guanylate cyclase and/or inactivation of phosphodiesterase remains to be determined.

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Characterization of single pacemaker channels in cardiac sino-atrial node cells

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Normal pacemaking in the mammalian heart is driven by spontaneously active cells located in the sino-atrial (SA) node. The rate of firing of these cells and the modulation of this rate by catecholamines are controlled by i_f , an inward Na- and K-current that turns on at voltages more negative than -40 mV^{-1} . The 'pacemaker' current i_f is also present in other types of cell where its ability to produce and modulate a depolarizing process may be useful¹⁰. For example, in vertebrate photoreceptors i_f drives the depolarization that terminates the light-induced hyperpolarization¹¹. Currents similar to i_f are also found in hippocampal neurones and DRG neurones^{12,13}. The present report shows for the first time that the opening of single i_f -channels of low conductance (1 pS) can be resolved using a modification of the patch-clamp technique¹⁴ on isolated SA-node cells. Modulation of i_f by adrenaline is shown to be mediated by an increase in the probability of channel opening, whereas the single-channel amplitude remains unchanged.

Although the 'pacemaker' current was one of the first currents to be discovered in cardiac tissues and has a mixed ionic nature which suggests a relatively large single-channel conductance such as that found for other non-specific cation currents^{15–17}, attempts at recording the activity of single i_f channels have previously been unsuccessful. This has led to the suggestion that either the elementary current steps are too small, or the current is carried by a transport mechanism that is not a channel¹⁸.

Fig. 1 Simultaneous measurements of whole-cell i_f and single i_f channels in a sino-atrial node cell. The bottom traces (labelled *d*) represent i_f recorded with a pipette in the whole-cell configuration during voltage-clamp steps from a holding potential of -32 mV. Voltages are labelled near corresponding traces. Activity from a membrane patch of the same cell was simultaneously recorded using an independent pipette in the cell-attached configuration. The top traces (*a* and *b*) are examples of patch recordings during i_f activation showing multiple step-like transitions. These transitions represent consecutive openings of single channels during hyperpolarization. Grids with fixed intervals were used to fit patch records by eye and to determine the elementary current step at each potential. These were -0.067 , -0.077 and -0.085 pA at -82 , -92 and -102 mV, respectively. The traces labelled *c* are averages of patch records ($n = 7$, 7 and 9 in *A*, *B* and *C*, respectively). Their time courses follow those of the corresponding whole-cell currents, indicating that patch activity reflects opening of single i_f channels. The maximum number of elementary openings was 3 at -82 mV, 5 at -92 mV and 6 at -102 mV. Notice that the time courses of mean-patch and whole-cell traces differ at -2 mV. This can be attributed to the different Na and K concentrations used for the patch-pipette and extracellular solutions (below) which determine different reversal potentials for the two traces. A similar observation applies to the data in Fig. 3.

Methods. Cells were dissociated from the sino-atrial region of rabbit hearts as described¹⁹. Their mean resistance and capacity were 1213 ± 417 M Ω (number of recordings (n) = 17) and 29.3 ± 9.5 pF (n = 20), respectively. The cells were superfused with warm (35 – 36°C) Tyrode solution containing (in mM): NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.8, glucose 10, hydroxyethyl piperazine ethane sulphonic acid (HEPES) 10 (pH 7.4). Single myocytes were whole-cell voltage-clamped using a pipette filled with intracellular-like solution containing (mM): NaCl 10, K-aspartate 130, MgCl₂ 1, EGTA 1, Na₂-ATP 2, HEPES 10 (pH 7.2). In experiments where adrenaline was used the pipette also contained 0.1 mM GTP, and ascorbic acid (0.5 mM) was added to the external solution. Pipettes for patch recording contained (mM): NaCl 70, KCl 70, MgCl₂ 1, CaCl₂ 1.8, BaCl₂ 1, MnCl₂ 2, HEPES 5 (pH 7.4). After a successful whole-cell recording of i_f was obtained in one cell, a second independent pipette was sealed onto the same cell for simultaneous cell-attached recording. Currents were recorded on tape and displayed on an 8-bit digital oscilloscope interfaced to a table computer. Single-channel currents were digitized at 500/1000 Hz and low-pass filtered before digital conversion using a 4-pole Bessel filter with a corner frequency of 80/160 Hz.

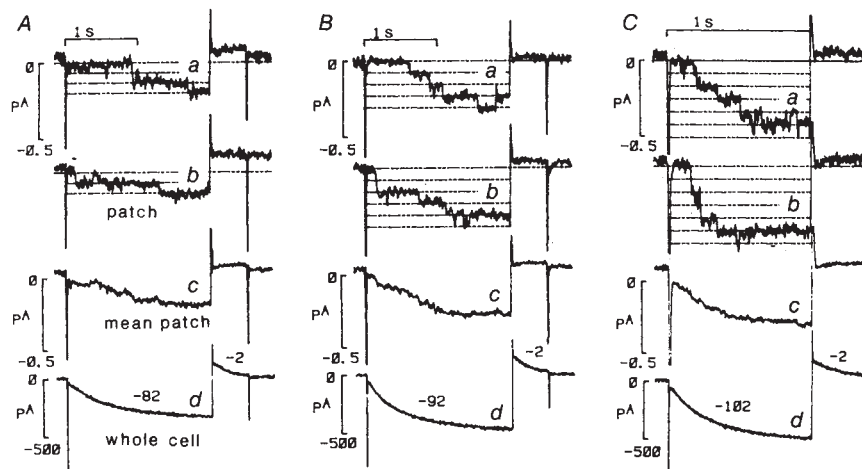
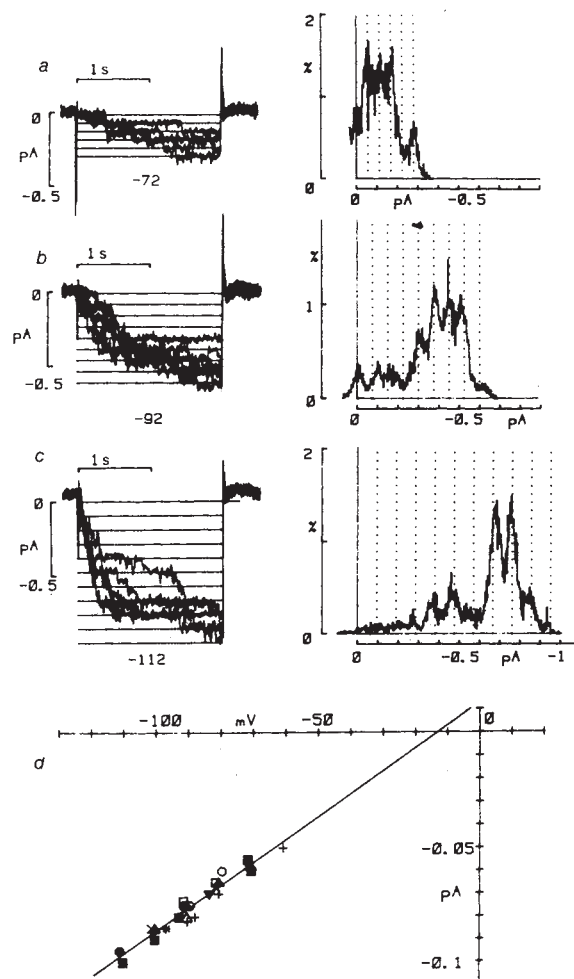


Fig. 2 *a*–*c*, transient distribution of patch currents during activation at different voltage levels; *d*, current-voltage relation for the single i_f channel. Whole-cell activation of i_f was obtained by applying 2 s hyperpolarizations to -72 (*a*), -92 (*b*) and -112 mV (*c*) from a holding potential of -32 mV through the pipette in whole-cell configuration. The left-hand panels show the superimposition of 4, 8 and 5 consecutive traces recorded from a membrane patch with a pipette in the cell-attached configuration sealed onto the same cell, during whole-cell hyperpolarizations. The right-hand panels show histograms of the current distribution for the same sets of patch records shown on the left. For each set of traces, the current axis was divided into intervals of 3 fA and the fraction of digitized points in each interval was plotted against current. Current levels corresponding to multiple channel openings can be clearly identified from the peaks in the histogram plots and correspond to single-channel levels evident in the current plots. Grids fitted by eye to histogram peaks (dotted lines) and current levels in the patch records (full lines) have interdistances of -0.056 pA (*a*), -0.075 pA (*b*) and -0.095 pA (*c*), representing the estimated single- i_f channel amplitudes at the absolute membrane voltages of -72 , -92 and -112 mV, respectively. The maximum number of channels opening increased with larger hyperpolarizations, varying from 6 at -72 mV to 9 at -92 mV and 10 at -112 mV. This reflects the increase in the degree of i_f activation observed in whole-cell conditions^{7,8}. *d*, Single-channel levels were measured in 10 cells by fitting either patch records or histograms as illustrated above in the voltage range -61 to -112 mV. Linear least-squares fitting of experimental points yields a single channel conductance of 0.98 pS and an extrapolated reversal at -13 mV.



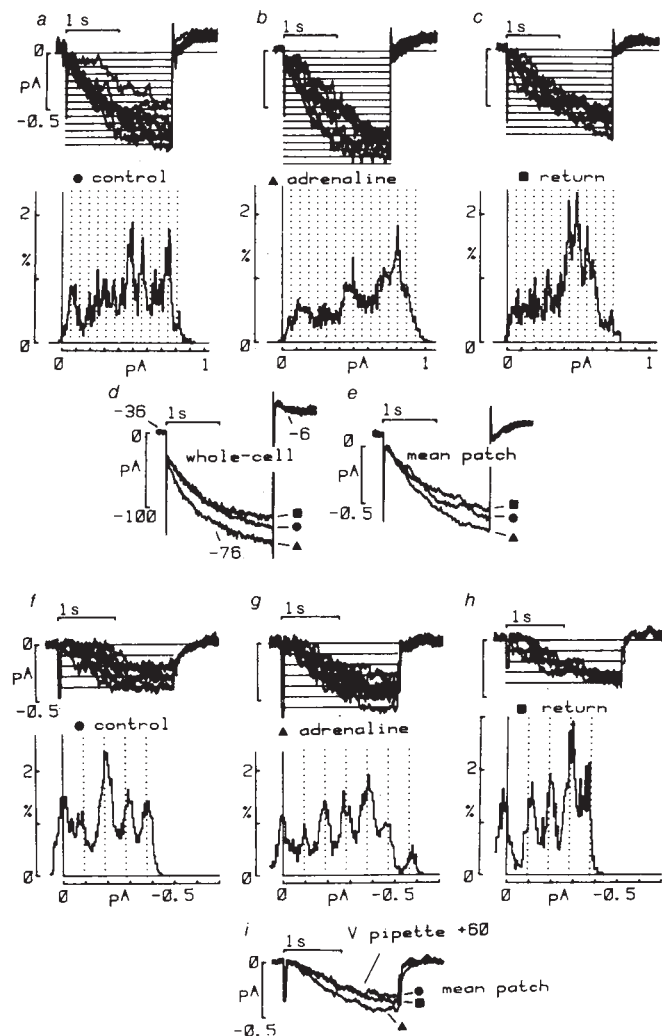


Fig. 3 Modulation of I_f channels by adrenaline. *a-e*, two-pipette experiment. *f-i*, single-pipette experiment. *a-e*, Whole-cell and patch currents were recorded during pulses to -76 mV from a holding potential of -36 mV in a control solution (*a*, $\bullet$), in a solution containing $5 \mu\text{M}$ adrenaline (*b*, $\blacktriangle$) and on return to the control solution after 2.5 min perfusion with the drug (*c*, $\blacksquare$). The single channel amplitude obtained from the fitting, done by eye, of patch current levels and histogram peaks did not vary in adrenaline (-0.062 pA). Adrenaline increased the maximal number of channels opened by hyperpolarizations (13 in *a*, 16 in *b* and 12 in *c*) and accelerated the rate of current development by decreasing the mean latency to first opening. For example, the mean time required for 8 channels to be open was 452 ($n=7$), 326 ($n=12$) and 481 ms ($n=6$) in panels *a*, *b* and *c*, respectively. This resulted in a faster activation rate of the mean patch current (*e*), which was comparable to that of the whole-cell current (*d*). Notice that the higher probability of channel opening favours the appearance of records with many open channels, and thus histogram peak levels corresponding to few open channels are depressed. Histograms were constructed from 7 (*a*), 12 (*b*) and 6 (*c*) records. *f-i*, Whole-cell pipettes were not used. Patch currents were recorded during 2 s, $+60$ mV pulses applied to the cell-attached pipette. Traces were electronically corrected for leakage and capacitive components and were stored after digital addition of the mean of 3 records during 2 s, -60 mV pulses. Previous control runs showed that adrenaline at the concentration used did not alter significantly either the resting level or the holding current in the cell (see for example panel *d*). As in the two-pipette experiments, addition of $5 \mu\text{M}$ adrenaline increased the maximal number of channels opening (from 4 to 6) and caused I_f channels to open more readily (for example, the mean time required for the sequential opening of 3 channels was 670 ($n=6$), 521 ($n=9$) and 791 ms ($n=5$) in *f*, *g* and *h*, respectively). However, as apparent from the superimposition of patch traces and histograms, the elementary current step was unchanged in adrenaline (-0.094 pA). Histograms were constructed from 7 (*f*), 9 (*g*) and 5 (*h*) records.

Recording of single-channel events of small amplitude during voltage pulses is complicated by the presence of capacitive transients and current drifts that cannot always be properly corrected. To overcome this problem two pipettes have been used on the same cell, one for whole-cell and one for cell-attached patch recording. Activation of I_f was obtained by applying intracellular hyperpolarizations via the whole-cell pipette as previously reported¹⁹. Although technically more complicated, the use of two pipettes on one cell has several advantages over the single pipette patch-clamp method: (1) voltage pulses delivered to the intracellular patch side do not involve charging of the cell-attached pipette, which eliminates the need for leakage and capacitive corrections and improves the resolution of single-channel current measurements; (2) single-channel activity and whole-cell currents are recorded simultaneously and can therefore be correlated; (3) the absolute membrane potential is known.

A recent investigation¹⁹ has shown that the current I_f in SA node cells has properties similar to those in the intact tissue. The bottom traces in Fig. 1 (labelled *d*) show whole-cell records of I_f activating during hyperpolarization to -82 , -92 and -102 mV in one cell. Simultaneous recording from a membrane patch of the same cell was performed using a second pipette in the cell-attached configuration, yielding records like those shown in traces *a* and *b*. At all voltages the patch records show step-like transitions consistent with sequential openings of single inward channels that occur simultaneously with the whole-cell-driven activation of I_f .

The elementary transitions that can be resolved in the cell-

attached current records are of small amplitude: -0.067 pA at -82 mV, -0.077 pA at -92 mV and -0.085 pA at -102 mV. The activity recorded in the membrane patch reflects opening of single I_f channels, as shown by the similar time courses of averaged patch records (traces labelled *c*) and corresponding whole-cell I_f records (*d*) at the three voltages investigated.

We studied the distribution of single open-channel current levels and determined the I_f elementary current step amplitudes during activation more accurately using the method shown in Fig. 2. The current histogram for several cell-attached traces (left-hand panels) recorded during repetitive whole-cell-driven membrane hyperpolarizations was constructed at each voltage. The histograms on the right give the fractional distributions of current levels for each set of traces. At all voltages the histograms peak at the denser regions in the current panels, which represent the overlapping single-channel current levels. Histogram peaks can be satisfactorily fitted by vertical grids with intervals of -0.056 pA at -72 mV, -0.075 pA at -92 mV and -0.095 pA at -112 mV. In the lowest panel (*d*) the single-channel curve of current against voltage was reconstructed in the limited voltage range where measurements can be made using the experiments shown in Figs 1 and 2 and 8 further experiments. The relationship is linear and the single channel conductance is found to be 0.98 pS. The extrapolation crosses the voltage axis at -13 mV, a value close to that expected at the same external Na and K concentrations for I_f in Purkinje fibres⁸.

Binding of catecholamines to cardiac β -receptors modulates pacemaker activity through the current I_f ^{3,10}. Pacemaking acceleration is caused by a shift of the current activation curve

to more positive voltages and is therefore attributable to an action of catecholamines on the i_f current kinetics^{1,2,19,20}. The effect of adrenaline on single i_f channels was investigated (Fig. 3). The upper panels (a-e) show an experiment where adrenaline 5 μ M was added to the cell superfusing solution. In agreement with previous results¹⁹, adrenaline caused the whole-cell i_f to increase during pulses to the current half-activation voltage range (panel d). A similar increase was also observed for the average of several patch records (e). Inspection of the patch records and corresponding histograms reveals that these changes are brought about by an increase of the maximal number of open channels (from 13 in a to 16 in b) and a decrease of the mean latency to first openings. The single-channel amplitude was, on the other hand, unaltered (-0.062 pA throughout). These changes are in agreement with the shift in the current activation curve mentioned above.

As shown in a recent study on i_f in SA node cells¹⁹, part of the effectiveness of adrenaline on i_f may be lost due to buffering of cell content by the whole-cell pipette solution. To avoid this, experiments were performed using cell-attached single-pipette patch-recording, thus excluding internal cell dialysis. Cell-attached patch records obtained with the single-pipette arrangement needed to be corrected for leakage and capacitive components, and single i_f channels could be clearly resolved only in a few cases. The lower panels in Fig. 3 (f-i) illustrate one such case where in control conditions (f) four channels of -0.094 pA were seen to open during +60 mV pulses applied to the cell-attached pipette. Perfusion with adrenaline (5 μ M, g) caused more channels (6) to open more quickly compared with control conditions, without any alteration in the elementary current step size. This resulted in a larger mean-patch current developing faster during a pulse (panel i), in agreement with the results of the two-pipette experiment above.

The data presented here show that under favourable experimental conditions the opening of low-conductance single pacemaker channels during whole-cell driven activation of i_f can be resolved in isolated SA node cells. In 70 mM KCl, 70 mM NaCl external solution the single i_f channel conductance is about 1 pS, a value much lower than that of other cation channels of low selectivity²¹.

The recording of single i_f channels will be useful in detailed investigations of the properties of the pacemaker current, and in particular of the way the current is regulated by the adrenergic neurotransmitter. Stimulation of cardiac β -adrenoceptors triggers a cascade of intracellular reactions leading to modifications of, among others, the slow Ca current and the pacemaker current^{1,3,22-25}, which are responsible for the chronotropic and inotropic responses of cardiac cells. There is now ample evidence that modulation of cardiac Ca channels involves phosphorylation by the cAMP-dependent protein kinase²⁶⁻³⁰. On the other hand, detailed information on the mechanisms involved in the catecholamine-controlled regulation of the current i_f has been limited so far by the absence of single i_f channel measurements. The data presented here indicate that the pacemaker current modulation by adrenaline is mediated by an increased probability that i_f channels open upon hyperpolarization, while the channel conductance is unaltered. These data confirm that adrenaline acts on the current kinetics rather than on its amplitude^{1,2,20}.

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Atrial natriuretic peptide causes pre-glomerular vasodilatation and post-glomerular vasoconstriction in rat kidney

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Atrial natriuretic peptide (ANP) can be extracted from rat hearts, and is found to increase fluid excretion by the kidneys when injected into test animals¹. The mechanism of ANP action is still unclear. ANP may reduce sodium reabsorption in the renal tubules, but it is also known that it increases the rate of glomerular filtration in the kidney, and relaxes preparations of smooth muscle, including one made from arteries that supply the kidney^{2,3}. To clarify its mode of action, we have studied directly the effects of semi-purified and synthetic ANP on blood vessels in the kidney of anaesthetized rats. We found that ANP causes a vasodilatation of the blood vessels which supply the glomeruli and a vasoconstriction of the arterioles which drain them. This substantiates the finding that increased filtration pressure participates in the natriuretic response⁴.

The lack of effect of ANP on sodium transport *in vitro* suggests that reduction of sodium reabsorption *in vivo* may be secondary to another process, perhaps to renal vasodilatation. Renal vasodilators cause natriuresis⁵. ANP both lowers the vascular resistance of the pre-constricted isolated perfused kidney and increases renal blood flow (RBF) in intact animals^{6,7}. Suprarenal aortic clamping prevents the rise of GFR on ANP administration and blunts the natriuretic response, suggesting that vascular events are involved in this response⁸.

However, RBF has also been found to be unaffected or even reduced after ANP administration^{9,10}. Since these conflicting effects could be due to different actions of ANP on distinct segments of the renal vascular tree, we investigated the effect of the peptide on pre- and post-glomerular resistance vessels.

For this purpose semipurified ANP (see legend to Fig. 2) or synthetic peptide (α -hANP) was infused intravenously into rats with unilateral hydronephrosis (Fig. 1, refs 11 & 12). Experiments were also performed in which synthetic ANP (rANP, 26 aminoacids) was introduced into the lucite chamber holding the

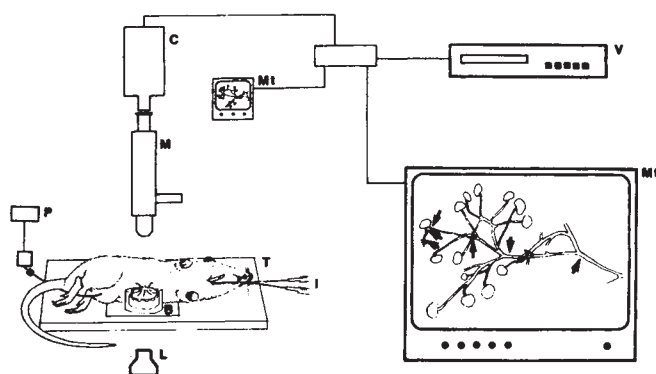


Fig. 1 Schematic representation of the experimental set-up. L, light source; T, automatic controlled temperature table; B, temperature-controlled kidney bath; P, blood pressure transducer linked to amplifier and recorder; M, microscope; C, videocamera; V, video recorder; Mt, monitors. The arrows on the monitor show the measuring points.

Methods. For intravital microscopy a unilateral hydronephrosis was induced by occluding the renal artery (60 min) and ligating the ureter of female Wistar-Furth rats. Seven to twelve weeks later the animals, which had been deprived of food but not of water for 15 h, were anesthetized with 5-ethyl-5-(1-methylpropyl)-2-thiobarbituric acid (Inactin, Byk Gulden, Konstanz, FRG; 100 mg kg⁻¹, i.p.). Body temperature was kept at 37 °C by a feedback-controlled heated operating table. The trachea was cannulated. Blood pressure was recorded by means of a strain gauge connected to a catheter in the left carotid artery. Infused solutions were delivered through two catheters into the left jugular vein. The hydronephrotic kidney was exposed through a flank incision and split at its greater curvature using a cautery knife. The dorsal sheet was retracted and the ventral one sutured to a semicircular wire holder. This was fixed to a lucite chamber filled with warmed (37 °C) isotonic and isocolloidal solution (Haemacel, Behringwerke, Marburg, FRG). The place at which the renal vessels entered the chamber was sealed with high vacuum grease (Dow Corning, Seneffe, Belgium). A vascular pathway that could be followed from the arcuate artery to the efferent arteriole was selected for study. Measurements were made on the arcuate arteries (vessels which do not give rise to afferent arterioles) and on the interlobular arteries (vessels which do give rise to afferent arterioles), as well as on the afferent arteriole close to and distant from the glomerulus and on the efferent arteriole close to the glomerulus and at the welling point, by two independent observers. Total magnification was 1389× and 2083×, respectively. Isotonic NaCl was infused throughout the experiment at 3.75 ml h⁻¹. An equilibration period of 2 h was allowed before starting the experiment.

kidney. Images of intrarenal vessels were formed in a television camera, through a microscope immersion objective, and displayed on monitors for on-line measurements of the lumen diameter. Each experiment was recorded on video tape for off-line analysis. The diameter of a given vessel, measured during an infusion of ANP or after application of the peptide to the lucite chamber holding the kidney, was expressed as a percentage of the diameter measured during the control period. The data was assessed by analysis of variance and the statistical significance evaluated by Wilcoxon's test for paired differences. Results are given as mean ± s.e.m.

A dose-dependent vasodilatation of the arcuate and interlobular arteries was observed during the infusion of semipurified ANP (Fig. 2, Table 1). The vascular diameter was 141% and 159% of control with the highest dose respectively, indicating a decrease in vascular resistance to less than 30% and 20% of control respectively. The proximal end of the afferent arteriole was also vasodilated by ANP (Fig. 2). The effect (137% of control with the highest dose infused) was, though significant, somewhat lower than that seen in the interlobular artery. Semipurified low molecular weight ANP had no effect on the afferent

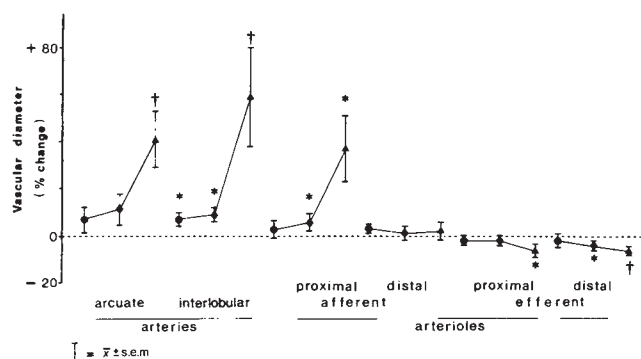


Fig. 2 Percentage change of the vascular diameter at different levels of the renal vasculature. Control measurements were made at the end of a 20 min i.v. infusion of potassium phosphate buffer (0.1 M, pH 7.4) at 10 or 100 µl min⁻¹. Semipurified ANP (see below) was infused for 20 min at the rate of ●, 1; ◆, 10 and ▲, 100 µl min⁻¹. (Statistical significance is indicated thus: *, *P* < 0.05; †, *P* < 0.02).

Methods. ANP was prepared from right and left atria dissected out of 44 rat hearts obtained under pentobarbital anesthesia (60 mg kg⁻¹, i.p.). The tissue was blotted, frozen in liquid nitrogen and pulverized. Boiling water (32 ml) was added to the 3.2 g powder obtained. After 15 min in a boiling water bath, the precipitate (11,000 g, 60 min, 4 °C) was discarded while the supernatant was removed and lyophilized. The residue was dissolved in 20 ml phosphate buffer (0.67 M, pH 7.4), cold acetone was added (90%) and the precipitate was separated by centrifugation (11,000g, 30 min, 4 °C). The remaining acetone in the precipitate was evaporated at room temperature under vacuum. The residue was dissolved in 10 ml 150 mM NaCl and stored frozen (-20 °C). After thawing, the atrial extract was separated by chromatography in 2 separate runs through a BioGel P60 column (50 × 2 cm). Elution was carried out with potassium phosphate buffer (0.1 M, pH 7.4, 2 °C) at 10 ml h⁻¹. The low molecular weight fractions containing natriuretic material were pooled. An aliquot (0.1 ml) of this pool increased sodium excretion by 69% in the rat bioassay²¹. Considering that atrial ANP content is 10–20 µg²² and that about 50% natriuretic activity is recovered in the low molecular weight fractions, our preparation should have contained about 100–200 ng ANP ml⁻¹ so that bolus injection of 0.1 ml should increase sodium excretion by 50–100%²³. The observed value was within this range (see above).

arteriole in the vicinity of the glomerulus. The efferent arteriole was constricted by the high dose near the glomerulus and by the middle and high dose away from it. The estimated increases in vascular resistance with the high dose of semipurified ANP was 35% near the glomerulus and 64% distant from it (Fig. 2). Blood pressure decreased from 112.1 ± 3.0 mm Hg during the control period to 102.1 ± 3.2 mm Hg (*P* < 0.01) during the 100 µl min⁻¹ ANP infusion. It remained unchanged (113 ± 5 and 109 ± 4 mm Hg) at 1 and 10 µl min⁻¹ ANP.

Infusion of synthetic α-hANP dilated all preglomerular vessels and constricted the efferent arteriole (Fig. 3). The beginning of the afferent arteriole was the segment most strongly vasodilated, its diameter increasing by 12 ± 8%, 38 ± 13% and 42 ± 6% at infusion rates of 0.05, 0.1 and 0.2 µg α-hANP min⁻¹ respectively (*n* = 3). The distal portion of the efferent arteriole was vasoconstricted by 5 ± 0.3% and 5.9 ± 1% with 0.1 and 0.2 µg α-hANP min⁻¹ respectively. The blood pressure fell from 95 mm Hg during the control phase to 90 ± 4 mm Hg during the infusion of 0.2 µg min⁻¹ ANP. Addition of synthetic rANP to the lucite bath also induced a pre-glomerular vasodilatation and a post-glomerular vasoconstriction (Table 2) without affecting the arterial blood pressure.

Our results clearly show that ANP, of either natural or synthetic origin, has the capacity to induce vasodilatation in the preglomerular renal vessels. Our findings are in agreement with

Table 1 Diameter of intrarenal vessels (μm) during infusion of semipurified ANP

	Control phase	Treatment rate of infusion of ANP (μl ANP pool min^{-1})		
		1	10	100
Arcuate artery	25.0 $\pm$ 1.6	26.6 $\pm$ 1.6	27.6 $\pm$ 1.9	32.4 $\pm$ 2.1
Interlobular artery	11.7 $\pm$ 1.2	12.5 $\pm$ 1.3	12.7 $\pm$ 1.3	18.1 $\pm$ 1.3
Afferent arteriole, proximal end	6.6 $\pm$ 0.9	6.6 $\pm$ 0.8	6.9 $\pm$ 0.8	9.5 $\pm$ 1.0
Afferent arteriole, distal end	6.0 $\pm$ 0.6	6.2 $\pm$ 0.6	6.1 $\pm$ 0.6	6.2 $\pm$ 0.7
Efferent arteriole, proximal end	12.4 $\pm$ 1.0	12.1 $\pm$ 0.9	12.4 $\pm$ 1.1	11.7 $\pm$ 1.0
Efferent arteriole, distal end	16.9 $\pm$ 1.7	16.3 $\pm$ 1.4	16.1 $\pm$ 1.5	15.2 $\pm$ 1.4

Semipurified ANP was prepared as described for Fig. 2. Each value is based on 7 measurements.

Table 2 Percentage change in vascular diameter in the hydronephrotic kidney after treatment with synthetic ANP

Location of measurement	Concentration of rANP in chamber (mol l^{-1})			
	10^{-9}	10^{-8}	10^{-7}	3×10^{-7}
Interlobular artery, near arcuate artery	+9.7 $\pm$ 2.9* (6)	$\pm$ 17.0 $\pm$ 7.4 (6)	+25.5 $\pm$ 7.3* (6)	+39.0 $\pm$ 11.9* (6)
Interlobular artery, near afferent arteriole	+3.6 $\pm$ 3.6 (6)	+12.5 $\pm$ 3.4* (6)	+27.6 $\pm$ 6.8* (6)	+31.6 $\pm$ 7.5* (6)
Afferent arteriole, near interlobular artery	+12.0 $\pm$ 3.6* (6)	+18.0 $\pm$ 3.8† (6)	+32.8 $\pm$ 7.7* (6)	+30.0 $\pm$ 4.6† (6)
Afferent arteriole, near glomerulus	+10.5 $\pm$ 2.6* (6)	+14.2 $\pm$ 2.9† (6)	+20.0 $\pm$ 3.9† (6)	+28.1 $\pm$ 7.9* (6)
Efferent arteriole, near glomerulus	2.9 $\pm$ 2.0 (12)	-5.5 $\pm$ 3.0 (12)	-10.0 $\pm$ 3.0* (18)	
Efferent arteriole, near welling point	-2.0 $\pm$ 3.0 (12)	-6.0 $\pm$ 5.0 (12)	-12.0 $\pm$ 3.0* (18)	

Statistical significance as follows: * $P < 0.05$, † $P < 0.01$. Measurements were made 15 min after adding rANP (R-R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y) to the lucite chamber holding the kidney. Figures in parenthesis indicate the number of measurements used to obtain each value.

the observation that ANP reduces the induced tone of isolated renal arcuate arteries from rat (2). We cannot exclude the possibility that in the hydronephrotic kidney the preglomerular vessels are partly vasoconstricted and thus more sensitive to vasodilatory stimuli. However, angiotensin ($0.2 \mu\text{g}$ per kg per min) still constricts preglomerular vessels by 30–40% in this system¹³. It has been suggested that the natriuretic effect of ANP depends mainly on enhanced GFR¹⁴. We have found in addition that this peptide increases proximal tubule stop-flow pressure (an index of filtration pressure, ref. 4). It appears therefore reasonable to conclude that in intact animals ANP also causes pre-glomerular vasodilatation. ANP may, in addition, induce post-glomerular vasoconstriction, and even the slight reduction in diameter seen with the lowest dose of ANP tested can cause a substantial rise in resistance. It has been shown that in the absence of vasoconstrictors ANP increases the renal vascular resistance and the glomerular filtration rate of the isolated perfused rat kidney, suggesting a preferential effect on the efferent arterioles¹⁵. Increased filtration fraction has been a repeated finding in studies of the renal effects of atrial peptides¹⁶.

The vasodilating effect of ANP might be due to activation of the particulate guanylate cyclase¹⁷. Involvement of dopamine cannot yet be excluded. Although dopamine antagonists like haloperidol block the natriuretic effect of ANP, they do not prevent the vasodilating effect of the peptide^{18,19}.

It seems likely that the primary role of ANP is to regulate renal haemodynamics, and that ANP-induced natriuresis depends on increased filtration pressure and dissipation of the medullary osmotic gradient¹⁰.

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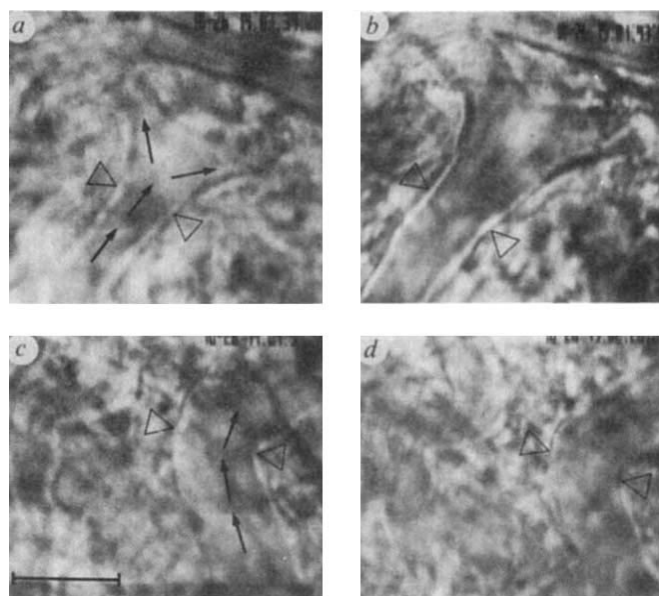


Fig. 3 Intravital microphotographs of split hydronephrotic kidney. *a*, Interlobular artery at the branching point into two afferent arterioles; *b*, same vessel after administration of 158 ng min^{-1} α -hANP; *c*, efferent arteriole just before the branching point, *d*, same vessel after the administration of 158 ng min^{-1} α -hANP. The flow direction is marked by arrows. Arrowheads, the same site of the vessel. Scale bar, $30 \mu\text{m}$.

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Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion

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In mammalian tissues the C-terminal amide structure has been found to occur only in neuroactive or hormonally-active peptides¹. About half known neuropeptide and peptide hormones have this unique chemical feature. Using a chemical detection method², a search for previously unknown peptides that possess the C-terminal amide structure in extracts of brain and intestine was carried out³ and a number of novel neuropeptides and hormonal peptides⁴, designated neuropeptide Y^{5,6}, PHI⁷, peptide YY⁸, galanin⁹ and neuropeptide K¹⁰ were isolated. We recently performed a similar search in porcine pancreas and found a high concentration of a peptide having a glycine amide at its C-terminus. Here we report the isolation, primary structure and biological activity of this novel peptide. The 49-residue peptide strongly inhibits glucose-induced insulin release from the isolated perfused pancreas and was therefore named pancreastatin. It may be important in the regulation of insulin secretion and in the pathogenesis and treatment of diabetes mellitus.

The pancreastatin-containing fractions were located by the amounts of glycine amide released from tryptic digests of the samples using the chemical method described previously². The peptide was extracted from porcine pancreas (20 kg) as described¹¹, and purified by ethanol fractionation, gel filtration (Sephadex G-25) and ion exchange chromatography (CM cellulose) (Fig. 1, legend). The final HPLC purification steps (Fig. 1a-c) yielded a homogeneous peptide preparation (0.2 mg) which was subjected to structural analysis.

Terminal analyses indicated that the peptide has an N-terminal glycine and a C-terminal glycine amide. From amino acid analysis, it consists of 49 residues: Ala (8), Arg (3), Asp

Table 1 Effect of pancreastatin and its fragments (10 mM) on glucose-induced insulin response of the isolated rat pancreas

Conditions of perfusion	n	Insulin release ($\mu\text{U min}^{-1} \text{ml}^{-1}$)	
		1-5 min	5-20 min
control	5	1,764 $\pm$ 210	227,184 $\pm$ 2,515
pancreastatin	6	676 $\pm$ 74†	21,134 $\pm$ 2,288
fragment 33-49	5	817 $\pm$ 178†	15,423 $\pm$ 3,524*
fragment 14-49	5	627 $\pm$ 116†	17,056 $\pm$ 3,114*

† $P < 0.01$, ‡ $P < 0.001$ v.s. controls.

* $P < 0.05$.

(1), Glu (13), Gly (9), His (1), Leu (1), Lys (2), Met (1), Phe (1), Pro (5), Ser (1), Thr (2), Trp (1). Edman degradation of the intact molecule (2 nmoles) using a gas-phase sequencer (Applied Biosystems) revealed the sequence of the first 25 residues of the N-terminal region. Because there are only two lysine residues in the molecule, the intact peptide (1.8 nmoles) was treated with a lysine-specific protease (Endoproteinase Lys-C, Boehringer, Mannheim) and the resulting digest was separated on HPLC (Fig. 1d). Peak I was found to contain a peptide with an N-terminal threonine. Sequence analysis indicated that this peptide corresponded to the middle fragment (residues 14-25) of the parent molecule. Peak II was found to contain two peptides, one with an N-terminal glycine and the other with an N-terminal glycine and a C-terminal glycine amide, indicating that this peak contained both the N- and C-terminal fragments of the parent molecule. Edman degradation of the peptides in peak II therefore yielded two residues for each cycle. Because the structure of the N-terminal fragment (residues 1-13) was already known, the structure of residues 26-49 was deduced from the sequence data by subtraction of the N-terminal sequence. The complete amino-acid sequence of porcine pancreastatin was deduced in this way (Fig. 2). In agreement with the proposed structure, pancreastatin was found to have a chemical molecular weight of 5103.1 daltons (calculated 5103.46) as measured by FAB mass-spectrometry (VG-ZAB-SE, R700, 10 μg pancreastatin in thioglycerol, mass range 5120-4780 u, scan rate 200 s/d, 15 scans accumulated).

The primary structure of pancreastatin is different from all other known peptides although the peptide has partial structural similarities to several known peptides. For example, pancreastatin shares the -Glu-Glu-Glu-Glu-Glu-structure (residues 34-38) with gastrin¹², and the C-terminal -Arg-Gly-NH₂ structure with vasopressin¹³. However, no other structural homology with these peptides is evident. These results suggest that pancreastatin may belong to a hitherto unknown peptide family.

Chemical synthesis of pancreastatin and its fragments was carried out using a solid-phase synthesizer. Fully protected pancreastatin (1-49) and the (14-49) and (33-49) fragments were synthesized by a published method¹⁴. After complete deprotection and cleavage by hydrofluoric acid, the crude synthetic preparations were purified by semipreparative HPLC. The synthetic pancreastatin (1-49) obtained was found to elute from the HPLC at the same position as the native peptide (Fig. 1). The results of amino-acid sequence analysis and molecular weight (found 5103.4) determination by FAB mass-spectrometry indicated that the synthetic peptide had an identical structure to native pancreastatin.

The effects of natural and synthetic pancreastatin and the synthetic fragments, pancreastatin (14-49) and pancreastatin (33-49) on glucose-stimulated insulin release, have been studied using isolated rat pancreas¹⁵. As expected, perfusion with glucose (16.7 mM) induced a biphasic insulin release from the isolated pancreas. Pancreastatin and the two fragments (10 nM) markedly decreased the early phase of insulin release (Fig. 3 and Table 1). The effect on late-phase insulin secretion was less

pronounced, but statistically significant for the fragments. Thus pancreastatin may have an important role in the regulation of insulin secretion. The potent effects of the C-terminal fragments of pancreastatin on insulin release suggest that this portion of the molecule is important for the biological activity and that C-terminal active fragments of pancreastatin may occur *in vivo*.

It has been suggested¹⁶ that suppression of insulin release upon glucose stimulation, especially in the early phase, is a characteristic feature of manifest type-2 diabetes. Because pancreastatin strongly suppresses insulin release, particularly in the early phase, it may be important to examine whether abnormalities in the regulation or action of pancreastatin and its receptors are involved in the pathogenesis of type-2 diabetes. If so, the discovery of pancreastatin would have therapeutic implications, particularly in the treatment of diabetes mellitus.

Preliminary results indicate that pancreastatin also inhibits the release of somatostatin upon glucose stimulation from the perfused pancreas. Pancreastatin may thus also be important in the control of carbohydrate metabolism and hyperglycaemia in diabetes.

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Glucocorticoid receptors lacking hormone-binding activity are bound in nuclei of ATP-depleted cells

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The glucocorticoid receptor binding capacity of rat thymus cells disappears when the cells are depleted of ATP by anaerobiosis, and rapidly reappears when ATP levels are restored¹⁻³. Loss and recovery of binding capacity occurs even when protein synthesis is suppressed with cycloheximide^{3,4}. In view of this and similar work in other cell systems^{5,6}, we proposed that in cells deprived of ATP the receptor is present in a form—the 'null receptor' form, as we shall call it—that cannot bind hormone⁴. Although many subsequent observations support this idea, no direct evidence has appeared for the existence of the null receptor. We have attempted to detect the null receptor in WEHI-7 mouse thymoma cells with a monoclonal antibody to the glucocorticoid receptor. Here we report that the null receptor is bound in the nuclei of ATP-depleted cells, and is present in amounts comparable to those of receptors in normal cells.

WEHI-7 cells incubated without hormone at 37 °C with 1 mM dinitrophenol (DNP) rapidly lose ATP and receptor binding capacity (Fig. 1). ATP levels drop to ~10% of control values within 10 min, whereas glucocorticoid receptor binding capacity in the cell cytosol decreases more slowly. No significant decreases occur in control cells incubated without DNP. These results are consistent with those obtained with other cells and other methods for lowering ATP¹⁻⁶. Receptor binding measured with whole cells falls similarly¹⁻⁶, and, as we show below, no binding is detectable in nuclei from the DNP-treated cells.

Although there is controversy over the intracellular location of glucocorticoid receptors in the absence of steroid⁷⁻⁹, by most cell fractionation procedures (including ours) the receptors without bound ligand are recovered in the cytosolic fraction. Because our experiments were conducted without hormone, we first looked for the null receptor in that fraction. Our attempts to immunoprecipitate receptors from cytosolic fractions from cells incubated with DNP for 90 min were unsuccessful, leading us to suspect that the null receptor, if it existed at all, was located

in the nuclear fraction. DNP-treated WEHI-7 cells were lysed using a freeze-thaw procedure by which receptors from cytosolic and nuclear fractions can be purified separately. The relative amounts of receptor in the two fractions were determined from the relative amounts of immunoreactive protein detected by Western blot analysis¹⁰.

Lanes 1 and 2 of Fig. 2 represent cytosolic and nuclear fractions from cells lysed immediately after the addition of DNP. Almost all the receptors (arrow) are located in the cytosolic fraction. The small amount of receptor in the nuclear fraction (1,800 c.p.m. ¹²⁵I in lane 2 compared with 15,000 c.p.m. in lane 1) may represent trace contamination with cytosolic receptor. This result is similar to that obtained with WEHI-7 cells in the absence of steroid and DNP. However, after 90 min of DNP treatment (Fig. 2, lanes 3 and 4), the receptors are located predominantly in the nuclear fraction (lane 4), even without hormone present. The cytosolic fraction (lane 3), which contained ~20% of the original binding capacity (Fig. 1b), contained a similar proportion of the immunoreactive receptor protein (5,000 c.p.m. in lane 3 compared with 21,000 c.p.m. in lane 4). The total amounts of receptor present in the cells before and after DNP treatment were comparable (17,000 c.p.m. in lanes 1 and 2 and 26,000 c.p.m. in lanes 3 and 4; the greater amount of receptor after DNP treatment probably indicates more efficient recovery of receptor from the nuclei than the cytosol). The receptors in the DNP-treated cells do not appear to be bound to the outer nuclear membranes as they were still present in nuclei treated with 0.05% Triton X-100 for 5 min at 3 °C to strip off the outer nuclear membranes¹¹ (23,000 c.p.m. in lane 5; 21,000 c.p.m. in lane 4). Preliminary studies, in fact, suggest that the null receptor is less easily extracted from nuclei than most nuclear-bound complexes formed by glucocorticoids.

In separate experiments (not shown), nuclei of DNP-treated cells were incubated for 2 h at 3 °C with 50 nM ³H-triamcinolone acetonide, in the presence and absence of 2 μM of the unlabelled compound, to test the ability of these receptors to bind hormone. No saturable binding was detected, indicating that the null receptor cannot bind steroid.

Nuclear binding of steroid hormone receptors in whole cells has until now been induced only with hormone, which forms receptor complexes that become activated and bind to the nucleus. New light may be shed on these processes by our observation that nuclear binding can be induced without hormone. Initially we proposed that the null receptor represents a stage of a cycle, possibly driven by cyclic phosphorylation-dephosphorylation⁴, in which the receptor, after binding in the

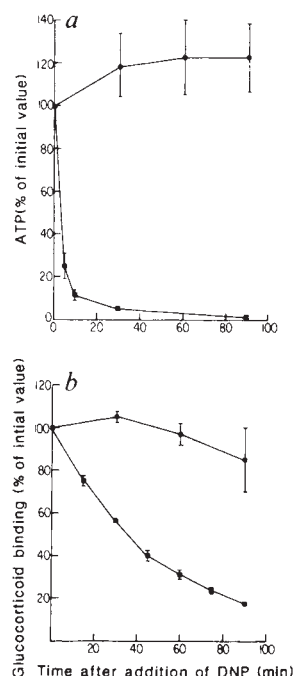


Fig. 1 Time course of drop in cellular ATP and glucocorticoid-binding capacity in WEHI-7 cells. WEHI-7 cells ($\sim 2 \times 10^8$), maintained in culture as described¹⁰, were washed by suspending in 80 ml of modified Krebs-Ringer buffer containing 25 mM hydroxyethyl piperazine ethane sulphonic acid (HEPES), pH 7.4, 3.6 mM sodium bicarbonate, 2 mg ml⁻¹ bovine serum albumin, at 37 °C for 5 min, the suspension centrifuged at 400g for 3 min, and the cells resuspended in 3.6 ml of the same buffer containing 1 mM DNP (■) or no DNP (●). Cells were incubated at 37 °C in an orbital water bath. At various times, aliquots of the cell suspension were assayed for ATP (a) and saturable glucocorticoid-binding capacity (b). Results are presented as % of initial value determined at 0 min, just after the addition of DNP, and represent the main value $\pm$ s.e.m. of duplicate measurements from two separate experiments. Cell viability, as determined by Trypan blue exclusion, was unaffected by the 90 min DNP treatment.

Methods. a. Cellular ATP was assayed by a modification of the luciferase procedure¹⁶. 25 μ l of the cell suspension was added to 500 μ l of 5% perchloric acid at 0 °C. After 30 min, the sample was centrifuged at 11,000 g for 4 min. Aliquots (450 μ l) of the supernatant were added to 500 μ l of a 1:1 mixture of trichlorotrifluoroethane and tri-*n*-octylamine¹⁷. The mixture was vortexed and centrifuged at 1,500 g for 2 min in a clinical centrifuge at room temperature. The amount of ATP in 20 μ l aliquots of the top phase, which contained the nucleotides in acid-free solution, was determined in a Packard liquid scintillation spectrophotometer from the light emitted upon addition of 100 μ l of a solution of desiccated firefly tails (Sigma) prepared in sodium arsenate according to the manufacturer's instructions. Absolute values of ATP were determined from a standard curve of events per 0.1 min versus known quantities of ATP prepared from a 2 mM stock solution. b. Glucocorticoid receptor binding capacity was assayed in cytosolic fractions obtained by a modification of the freeze-thaw procedure¹⁰ in which 100 μ l of the cell suspension was added to 250 μ l of lysis buffer containing 10 mM trimethylamino-ethanesulphonic acid (TES), pH 7.6 at 0 °C, 50 mM NaCl, 1 mM EDTA, 20 mM sodium molybdate and 10% glycerol (v/v). After the broken cell suspension had been centrifuged at 11,000g, aliquots of the cytosolic fraction were incubated with ~ 50 nM ³H-triamcinolone acetonide (specific activity = 43.8 Ci per mmole) in the presence or absence of 2 μ M triamcinolone acetonide to determine glucocorticoid receptor binding capacity¹⁸.

nucleus as the activated hormone/receptor complex and transducing hormonal activity, is released in the null form and the hormone binding form is subsequently regenerated. It has been shown that the receptors for glucocorticoid and other steroids are phosphorylated and lose hormone-binding activity under

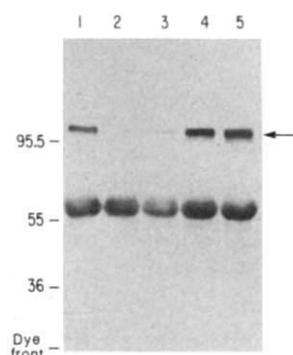


Fig. 2 Cellular localization of the glucocorticoid null receptor. Samples shown are: cytosolic (lane 1) and nuclear (lane 2) fractions of WEHI-7 cells removed at time = 0 min, immediately after addition of DNP; cytosolic (lane 3) and nuclear (lane 4) fractions of WEHI-7 cells exposed to 1 mM DNP for 90 min; and Triton X-100-washed nuclear fraction of WEHI-7 cells exposed to 1 mM DNP for 90 min (lane 5). At various times after the addition of DNP, 1 ml of the WEHI-7 cell suspension was centrifuged at 400g for 1.5 min. The cell pellet was lysed by a freeze-thaw procedure¹⁰ in 1 ml of a lysis buffer containing 10 mM TES, pH 7.6 at 0 °C, 50 mM NaCl, 2 mM MgCl₂, 20 mM sodium molybdate and 10% (v/v) glycerol. After lysis, the broken cell suspension was centrifuged at 400g at 3 °C for 3 min. The supernatant was used without modification as the cytosolic fraction. The nuclei were suspended in 5 ml of the lysis buffer, centrifuged at 400g for 5 min, and the supernatant discarded. The nuclei were then resuspended in 600 μ l of lysis buffer. Nuclei to be treated with Triton X-100 were suspended in 5 ml of 0.05% Triton X-100 in 6 mM MgCl₂ for 5 min at 3 °C, centrifuged at 400g for 5 min, the supernatant discarded and the pellets resuspended in 600 μ l of lysis buffer. Glucocorticoid receptors were denatured in both fractions by addition of 200 μ l of a detergent solution containing 10 mM TES, pH 7.6 at 0 °C, 10% (w/v) SDS, 10 mM sodium molybdate and 10% (v/v) glycerol, and heating the samples in a boiling water bath for 5 min (cytosolic fraction) or 10 min (nuclear fraction). After cooling to room temperature, 2 ml of a 5% solution of Triton X-100 (w/v) in 10 mM TES, pH 7.6 at 0 °C, 10 mM sodium molybdate, 10% (v/v) glycerol was added to bring the total volume of each sample to 5 ml. Ascites fluid (10 μ l) of BuGR2 antibody (directed against the glucocorticoid receptor¹⁹) was added to each sample, and the receptors purified on protein A-Sepharose CL-4B columns¹⁰. Antibody-purified receptors were run on 7.5% polyacrylamide gels under denaturing and reducing conditions, transferred to nitrocellulose and identified by reaction with BuGR2 antibody and subsequent labelling with ¹²⁵I-sheep anti-mouse IgG (ref. 10). After autoradiography the amount of receptor in each sample was determined by counting the ¹²⁵I in the receptor-containing region (arrow). The sizes (daltons $\times 10^3$) and migration positions of pre-stained relative molecular mass standards (Diversified Biotech) are shown. The immunoreactive protein which migrates just above the 55,000 standard is BuGR2 antibody.

cell-free conditions when treated with phosphatase (see refs 12 and 13 for reviews). Because the null receptor can also be generated in cells in the absence of steroid^{3,6}, it can directly interconvert with the normal unoccupied receptor. Our demonstration that the null receptor is located in the nuclei of ATP-depleted cells, rather than in the cytosolic fraction, however, raises some interesting questions. Among these is the possibility that the null receptor may be present as the predominant form in some of the mutant cells which have been considered receptor-deficient based on the lack of binding capacity or receptor protein in their cytosolic fractions^{14,15}.

We do not know where the null receptor is bound in the nucleus, nor whether it can initiate biological activity. Many other questions also remain, such as how the null receptor is formed from the normal receptor; whether it interconverts with the normal receptor in metabolically intact cells; and whether its accumulation in nuclei of ATP-depleted cells means that

release of receptors from nuclei is an energy-requiring step. Our identification and localization of the null receptor should allow definitive experimental answers to these and other questions.

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A new subunit of the human T-cell antigen receptor complex

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The T-cell antigen receptor binds antigen in association with a cell surface molecule encoded by the major histocompatibility complex (MHC)¹. MHC restricted recognition of antigen by this receptor leads to the complex pattern of programmed gene expression that characterizes T-cell activation. The eventual understanding of human T-cell function will require the complete elucidation of the structure of the human T-cell antigen receptor. On human T cells, clonally determined, disulphide-linked α and β chains of the receptor are non-covalently and stoichiometrically associated with three additional polypeptides known as the T3 complex^{2,3}. These receptor subunits are glycoproteins of relative molecular mass (M_r) 25,000 (25K) and 20K (γ and δ) and a non-glycosylated 20K protein (ϵ). Our studies of murine T cells show that the mouse T-cell antigen receptor consists of at least seven distinct polypeptide chains⁴⁻⁶. In addition to clonotypic α and β chains, the murine complex consists of glycoproteins of 26K and 21K and endoglycosaminidase F (endo F)-insensitive polypeptides of 25K, 21K and 16K. The latter, which we have termed ζ (zeta), exists as a homodimer within the complex. The 26K component (gp26) has been shown to be the murine analogue of the human δ chain^{6,7}. Other cross species homologies remain to be established, however none of the described human receptor components appear similar to the murine ζ polypeptide. We report here the use of an antiserum raised against the murine ζ subunit to identify a previously unrecognized component of the human T-cell antigen receptor. This human protein is T-cell specific and biochemically similar to the murine ζ polypeptide.

Rabbit antisera were raised against the ζ chain after separation of receptor components by sodium dodecylsulphate polyacryl-

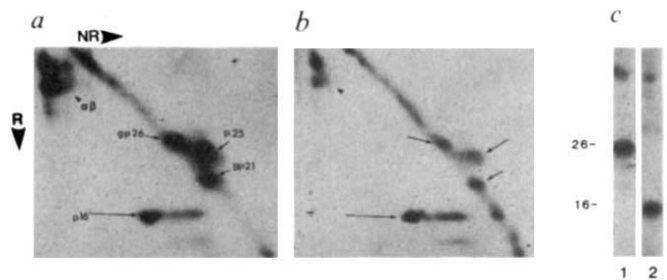


Fig. 1 Immunoprecipitation of radio-iodinated murine T-cell antigen receptor complex from the antigen specific hybridoma 2B4. Specific immunoprecipitates with *a*, the anti-clonotypic monoclonal antibody A2B4-2, or *b*, anti- ζ antiserum were subjected to two-dimensional diagonal non-reducing (NR)/reducing (R) electrophoresis. Arrows delineate subunits of interest: α - β are 40-45K glycoproteins; gp26 is a 26K glycoprotein (δ); p25 migrates at 22K under non-reducing and 25K under reducing conditions and gp21 is a 21K glycoprotein; p16 is the ζ chain. The 21K endo F insensitive protein, p21 (ref. 5) is not seen in this study. *c*, Solubilized cell membranes radio-iodinated using Iodobeads (Pierce) were immunoprecipitated and incubated with ionic detergents SDS and DOC before elution, reduction and SDS-PAGE. Lane 1, anti- δ rabbit serum; lane 2, anti- ζ rabbit serum.

Methods. 2B4 cells were maintained in exponential phase of growth as previously described¹². Lactoperoxidase catalysed radio-iodination with ¹²⁵I at 4 °C, lysis of cells in 0.5% Triton X-100 and immunoprecipitation using antibodies precoupled to protein A Sepharose (Pharmacia) were performed as described⁴. 2B4 cell membranes were prepared and solubilized as described⁵. Incubation of immunoprecipitates in ionic detergents SDS and DOC was carried out by the addition of 0.2-0.4% SDS and 0.2% DOC to protein A Sepharose immunoprecipitates after an initial wash in 0.1% Triton X-100 wash buffer. After 15 min at 22 °C the immunoprecipitates were again washed in 0.1% Triton X-100 containing wash buffer and analysed after reduction on 12.5-13% polyacrylamide gels using the buffer system of Laemmli¹⁶. Two-dimensional diagonal electrophoresis was carried out using 12.5% SDS-PAGE tube gels in the first dimension on non-reduced material. After reduction and equilibration in 0.5% dithiothreitol, 0.1% SDS and 0.125 M Tris, pH 6.8 for 2 h at room temperature, SDS-PAGE in the second dimension was carried out on 12.5% polyacrylamide slab gels (Hoeffer) according to the manufacturer's recommendations. Fixing of gels, glycerol treatment, drying and autoradiography were all performed as previously described⁴. Solubilised 2B4 cell membranes were used for the preparation of antisera. Insoluble material was removed at 15,000g and the supernatant was immunoprecipitated with A2B4-2 covalently linked to cyanogen bromide-activated Sepharose (Pharmacia). After extensive washing of immunoprecipitates using buffers described previously⁷, the receptor complex was eluted from the beads at 37 °C in a buffer consisting of 0.1% SDS, 50 mM Tris, pH 6.8, 0.5 M NaCl, 1 mM Phenylmethylsulphonylfluoride (Sigma), 10⁻¹⁰⁰ μ g ml⁻¹ leupeptin (Boehringer Mannheim), 10 μ g ml⁻¹ Aprotinin (Boehringer Mannheim), 10 mM EDTA and 10 mM iodoacetamide. After elution the material was concentrated at 22 °C using Centricon 10 Microconcentrators (Amicon). Radio-iodinated, solubilized A2B4-2 membrane immunoprecipitates were added to the concentrated material, boiled in 3% 2-mercaptoethanol/10% glycerol and electrophoresed on 17.5% polyacrylamide gels cross-linked with Acrylaide (FMC Corporation). After autoradiography of the undried gel, the 16K band was cut from the gel, eluted in 0.1% SDS 10 mM Tris, pH 7.6 at 95 °C and concentrated as above. Material from approximately 1.5 $\times$ 10¹⁰ cell equivalents was emulsified in complete Freund's adjuvant (CFA) in a final volume of 2 ml and injected into an adult female New Zealand White rabbit by multiple subcutaneous injections. The animal was boosted after 5 weeks with antigen emulsified in CFA and bled two weeks thereafter.

amide gel electrophoresis (SDS-PAGE). The antiserum was evaluated by immunoprecipitation of lysates from the murine T-cell hybrid 2B4. The proteins specifically immunoprecipitated from surface-iodinated cells by the anti-clonotypic monoclonal antibody A2B4-2 were compared to those precipitated by the

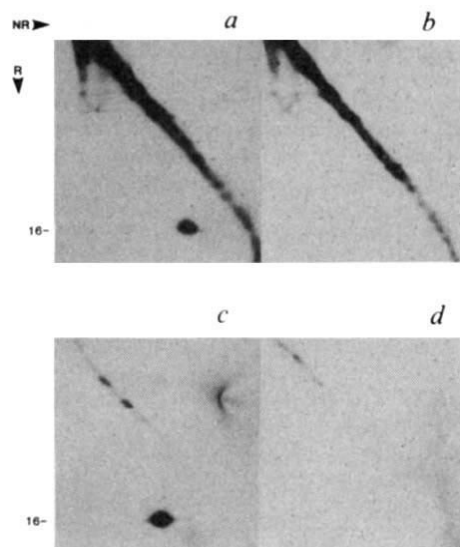


Fig. 2 Identification of the human ζ chain. Radio-iodinated solubilized membranes from Jurkat cells (*a, b*), human tonsillar T cells (*c*), and human tonsillar B cells (*d*) were immunoprecipitated with the anti- ζ serum (*a, c, d*) or non-immune rabbit serum (*b*) and subjected to electrophoresis on diagonal gels.

Methods. Jurkat human tumour cells were maintained as described previously⁴. Purified tonsillar T and B cells (provided by Dr John Kehrl) were prepared as previously described¹⁷. Tonsillar T cells were judged to be 90% pure by indirect immunofluorescence with the T-cell specific monoclonal antibodies OKT4 and OKT8. B cells were 94% surface immunoglobulin positive. In this study 6.5×10^7 T cells and 5×10^7 B cells were used.

anti- ζ serum on two-dimensional non-reducing/reducing diagonal gels (diagonal gels) (Fig. 1*a, b*). The serum specifically precipitated all of the components of the T-cell receptor complex seen with the monoclonal antibody. The relative intensities of the receptor components are different between panels *a* and *b*. This may be due to disruption of the complex by the anti- ζ serum or to the presence of some dimeric ζ not associated with the receptor complex. A 16K band present on the diagonal in the material precipitated by the antiserum may represent monomeric ζ . A small amount of streaking is seen at 16K and 14K in both panels; this finding is variable and probably due to proteolysis. This antiserum also immunoprecipitates an identical pattern of subunits from murine thymocytes (data not shown). In contrast, the anti- ζ serum fails to immunoprecipitate specific proteins from lysates of a murine B-cell tumour line LK 35.2, or from murine fibroblasts.

To identify the direct target antigen of this antiserum we subjected radio-iodinated immunoprecipitates to an incubation in a buffer containing the ionic detergents SDS and deoxycholate (DOC). This treatment results in dissociation of the receptor complex⁷. Immunoprecipitation from radio-iodinated membranes with an antiserum raised against the carboxy terminus of the murine δ chain⁷ revealed a single band at 26K when the SDS/DOC incubation was carried out (Fig. 1*c*). When the anti- ζ serum was used, SDS/PAGE under reducing conditions revealed a single prominent band at 16K. In this experiment a small amount of material is seen at 50K with both of the specific antisera and with control serum.

We used this anti- ζ serum to look for a similar protein in human T cells. The human T-cell tumour line Jurkat expresses both α - β and the T3 complex on its surface⁸. Solubilized membranes from these cells were radio-iodinated using lactoperoxidase and immunoprecipitates were electrophoresed on diagonal gels. Immunoprecipitation with the anti- ζ serum (Fig. 2*a*) revealed a 32K protein with a M_r of 16K after reduction. No other components of the T-cell antigen receptor complex

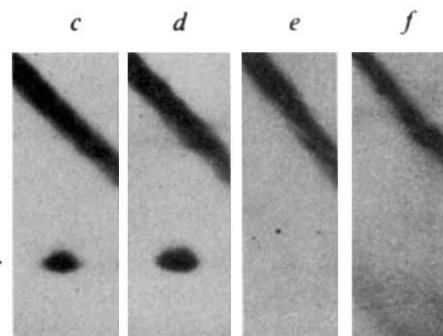
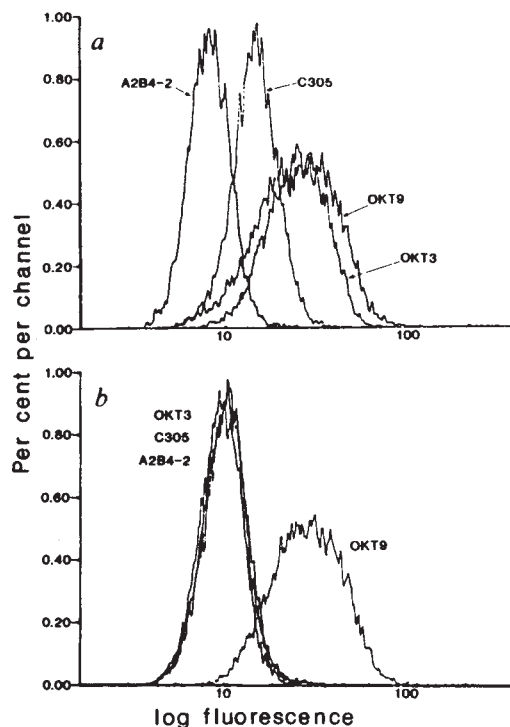


Fig. 3 Jurkat cells incubated for 24 h with *a*, A2B4-2; or *b*, OKT3 ascites at a dilution of 1:1000. The cells were washed 3 times and treated for 30 min at 4°C with ascites or culture supernatants of monoclonal antibodies A2B4-2, OKT3, OKT9 or C305. The cells were washed and incubated with fluorescein-conjugated Fab fragments from affinity-purified goat anti-mouse serum, then subjected to FACS analysis as described¹⁸. Incubation with OKT9 for 24 h resulted in persistence of surface antibody as measured by FACS, interfering with analysis of antibody binding. In parallel, Jurkat cells were surface iodinated after 24 h treatment with A2B4-2 (*c*), OKT9 (*d*) or OKT3 (*e, f*). Equivalent numbers of trichloroacetic acid precipitable counts were immunoprecipitated with anti- ζ serum (*c, d, e*) or non-immune serum (*f*) and subjected to electrophoresis on diagonal gels. The position of ζ is shown. The non-immune immunoprecipitate shown (*f*) is representative of similar control immunoprecipitations carried out after 24 h treatment with A2B4-2 or OKT9.

were detected with varying exposures of the autoradiograph. Non-immune serum (Fig. 2*b*) revealed a pattern of non-specific material on the diagonal similar to that seen in Fig. 2*a*, but the 16K dimer was clearly not present. Surface iodination of intact Jurkat cells gave identical results (data not shown). We prepared radioiodinated solubilized membranes from human tonsillar T and B cells to examine whether this protein is found in normal human T cells. The anti- ζ -antiserum immunoprecipitates a 16K protein with a non-reduced M_r of 32K from T cells but not from B cells (Fig. 2*c, d*). It is clear that in these cells no other components of the T-cell antigen receptor are specifically

precipitated with this antiserum. Additional studies revealed that the 16 K human homodimer has the same apparent pI as the murine ζ chain and is likewise insensitive to endo F (data not shown).

Thus ζ , which is clearly a component of the murine T-cell antigen receptor complex, has a human analogue which: (1) is immunologically related to the murine chain; (2) has identical size and charge characteristics to the murine protein and (3) is found in T cells but not B cells. Based on these findings we analysed radio-iodinated immunoprecipitates of Jurkat cells obtained by using the anti-T3 monoclonal antibody OKT3 (ref. 9) and the anticolonotypic antibody C305 (ref. 8). With both antibodies, material that migrated identically to ζ was consistently seen on long exposures of autoradiographs of diagonal gels (data not shown). Although the level of ^{125}I associated with this material was significantly less than that found in the other subunits of the complexes, these results provided suggestive evidence that the human ζ polypeptide is part of the T-cell antigen receptor.

One possible explanation for the apparently lower amount of ζ -like material in human receptor immunoprecipitates is that solubilization may lead to disruption of the complex. We wished to determine whether the human ζ analogue was associated with the other receptor components on intact cells. To test this, we used antibody-mediated co-modulation. This approach has allowed the determination of specific non-covalent association of membrane proteins. In particular it has been used to show the association of the T3 δ chain with the clonotypic α - β heterodimer on the surface of human T cells¹⁰. Modulation of surface T3 with anti-T3 reagents results in loss of both the clonotypic and T3 antigenic determinants without affecting other cell surface antigens. Since the anti- ζ serum does not bind to the surface of cells as measured by fluorescence activated cell sorter (FACS) analysis, we modified the standard co-modulation technique by carrying out surface iodination of cells at 4°C after antibody incubation. This labelling allowed us to quantify surface ζ by immunoprecipitation.

Jurkat cells were used for these studies because specific reagents for this cell line are available. Jurkat cells were treated for 24 hours with ascites from hybridomas producing the OKT3, OKT9 or A2B4-2 monoclonal antibodies to modulate antigen receptors. OKT9 is a monoclonal antibody which binds the human transferrin receptor¹¹. A2B4-2 does not bind to human cells¹² and served as a control. When T3 surface expression was measured by FACS, the A2B4-2 pretreated cells bound considerably more OKT3 than did the OKT3 pretreated cells (compare Fig. 3a with 3b). As expected, the α - β chains of the antigen receptor were co-modulated with the T3 antigen after OKT3 treatment as shown by the reduced binding of the anti-clonotypic C305 antibody. Immunoprecipitation of the surface iodinated cells with C305 confirms the FACS data; essentially no residual heterodimer is seen after T3 modulation, but OKT9 and A2B4-2 treated cells have approximately the same amount of heterodimer (data not shown). The association of ζ with the T-cell antigen receptor complex on the intact cell is shown by the loss of ζ chain expression from the cell surface on OKT3-treated cells (Fig. 3c-f). Thus ζ co-modulates with the α - β heterodimer and T3 δ confirming the *in situ* association of these polypeptides. The specificity of this co-modulation was demonstrated by FACS analysis. OKT3-treated cells showed no loss of surface transferrin receptor binding compared to A2B4-2 treated cells, nor was there loss of binding of an anti-LFA-1 antibody MHM-23 (ref. 13) (data not shown). In addition, when radio-iodinated OKT3-treated cells were compared to OKT9 or A2B4-2 treated cells on SDS-PAGE, few if any differences in the pattern of total cell surface proteins were seen.

Solubilization of multicomponent membrane complexes invariably results in a certain degree of denaturation of native protein-protein interactions, as shown in studies of the T-cell antigen receptor and the IgE receptor^{4,14}. Optimizing ratios of

lipid to detergent and the use of non-ionic detergents can minimize this denaturation¹⁵. In the T-cell antigen receptor system however, ζ , which can be co-immunoprecipitated in a consistent stoichiometric relationship with other subunits from mouse cells, is not similarly co-immunoprecipitated from human T cells. This disparity is probably due to minor structural differences that are manifest as varying degrees of disruption of subunit interactions upon solubilization.

Aside from the identification of a new human T-cell antigen receptor component, we have shown that: (1) when studying membrane protein complexes, comparison of the same receptor system in different species may allow for the identification of interactions that might not otherwise be detected; (2) because of the receptor denaturation that occurs on solubilization, methods other than co-immunoprecipitation, such as co-modulation and chemical cross-linking, may prove useful to help determine specific associations between cell surface structures.

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Athymic mice express a high level of functional γ -chain but greatly reduced levels of α - and β -chain T-cell receptor messages

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T lymphocytes differentiate and mature in the thymus. It is here that thymocytes with reactivities to self antigens are eliminated and those with specificities to 'altered' self-major histocompatibility complex (MHC) gene products are positively selected¹. The selections are presumably carried out on the basis of their T-cell antigen receptors (TcR). The genes of the α - and β -chain T-cell antigen receptors have been cloned²⁻⁷. A third T-cell specific gene capable of undergoing somatic rearrangement has also been identified⁸; the role of this third gene is not known. An order of expression of γ , β , then α is found during T-cell ontogeny^{9,10}. But although α - and β -chain messages are often functional¹¹⁻¹⁵,

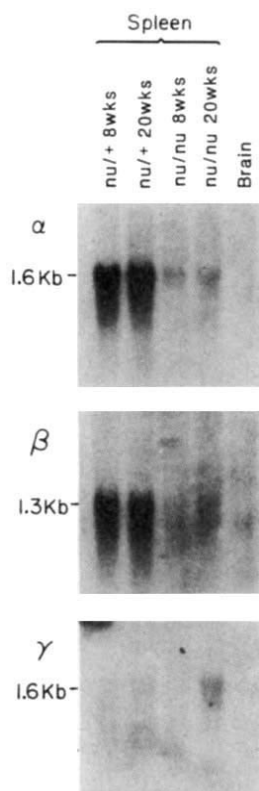


Fig. 1 Northern blot analysis of spleens in young and old nude mice. Congenitally athymic nude (*nu/nu*) mice on a BALB/c (10th backcross) background and their littermate (*nu/+*) controls were used under pathogen-protected conditions in the animal facility maintained by the Ontario Cancer Institute, Toronto. Original breeding pairs were obtained from Taconic Farms, N.Y. The life span of nude mice was 1 to 2 years under the maintenance conditions used. All nude mice used in these studies appeared in good health. RNA was extracted from spleens by the guanidine isothiocyanate and CsCl_2 gradient centrifugation procedures²⁹ and treated with glyoxal. Total RNA (30 μg per spleen) was electrophoresed through 1% agarose in 10 mM sodium phosphate buffer pH 7.0 and transferred to Gene Screen Plus (NEN). Hybridization was to ³²P-labelled nick-translated α (5/10-20 D α , HindIII-EcoRI fragment), β (5/10-20 D β , TaqI-EcoRI fragment) and γ (8/10-2 γ 1.1, AvaI-EcoRI fragment) constant probe³³. Following hybridization in 5 $\times$ SSC, 5 $\times$ Denhardt's 10% dextran sulphate and 0.1% SDS at 65 $^\circ\text{C}$, blots were washed three times in 2 $\times$ SSC, 0.1% SDS at 65 $^\circ\text{C}$ and once in 0.2 $\times$ SSC, 0.1% SDS at 65 $^\circ\text{C}$.

γ transcripts are rarely functional in thymocytes or mature T cells¹⁶⁻¹⁸. To define the sequential order of expression of these genes further and to continue the search for a possible role for the TcR γ gene products, we investigated the expression of 'functional' α -, β - and γ -chain transcripts in young athymic mice. These mice express an undetectable amount (less than one in 8×10^5 spleen messages) of 'full-length' α - and β -chain T-cell receptor transcripts, but an increased level of expression of 'full-length' γ chain messages. Nucleotide sequence analysis of four γ complementary DNAs show that all four γ transcripts sequenced are functional. These findings suggest that γ gene products may be important in a prethymic or extrathymic pathway and may represent a second type of T-cell recognition, possibly in a lineage in which α and β genes are not used.

To investigate the expression of the α , β and γ T-cell receptor messages in athymic mice, we extracted RNA from total spleen cells from mice with genotype BALB/c (*nu/nu*) at 8 weeks and 20 weeks of age. Control spleen RNA was also obtained from mice with genotype (*nu/+*) at 8 and 20 weeks of age. The level of expression of these T-cell receptor genes was evaluated using

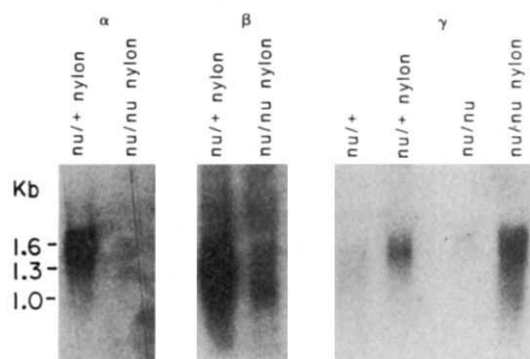
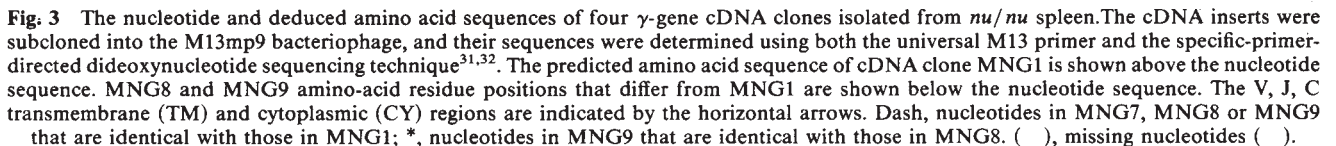


Fig. 2 Northern blot analysis of nylon wool-passed spleen cells from nude mice. Spleen cells were passed over nylon wool columns according to the method of Julius *et al.*²⁰. Recovery of spleen cells after nylon wool passage varied (15-20%). Total RNA (15 μg each) and blots were prepared as described in Fig. 1. Hybridization was carried out in 5 $\times$ SSC, 5 $\times$ Denhardt's, 10% dextran sulphate, 0.1% SDS at 65 $^\circ\text{C}$. Nick-translated constant probes of α , β and γ chain were used³³. After hybridization, blots were washed three times in 2 $\times$ SSC, 0.1% SDS at 65 $^\circ\text{C}$ and once in 0.2 $\times$ SSC, 0.1% SDS at 65 $^\circ\text{C}$.

Northern blots. A typical result is shown in Fig. 1. The experiments clearly show that a much lower level of the α - and β -chain T-cell receptor transcripts are found in both 8 and 20 weeks old athymic mice. However the same level of expression of γ -chain messenger RNA is found in BALB/c (*nu/nu*) mice and in control mouse spleen. Although the expression of these messages did not increase in old (20 weeks) control *nu/+* mice, a higher level of these messages is found in old athymic mice. The sizes of these γ -chain transcripts also suggest that they mainly consist of 'full length' 1.6 kilobase (kb) mRNA. We have constructed cDNA libraries derived from spleen cells of 8 weeks old *nu/+* mice and 8 weeks old athymic *nu/nu* mice. The cDNA clones with sequences homologous to the constant region of either α -, β - or γ -chain genes were isolated and counted. Results of one such experiment (Table 1) confirm that very low levels of α - and β -chain messages are found in the athymic mice whereas the level of γ chain messages is near normal.

The above data reflect the total amount of α -, β - or γ -chain T-cell receptor gene transcripts in total spleen RNAs. However, it is known that spleens from athymic mice have a low number of T cells (reflected by the level of Thy-1⁺ cells¹⁹). We enriched for Thy-1⁺ spleen cells from 8-week-old *nu/nu* spleens using nylon wool²⁰ and compared them with the nylon-wool purified cells from spleens of mice with genotype *nu/+* at the same age (Fig. 2). The cells from athymic mice express a much lower level of both the α - and β -chain TcR transcripts. Note also that, although control T cells express a high level of full-length transcripts of the α and β messages (1.6 and 1.3 kilobases (kb), respectively), cells from the athymic mice do not contain a high proportion of full length message. This is particularly evident in the β -chain transcripts, where we find a prominent band of truncated message at 1.0 kb. Examination of the sizes of eight inserts from the cDNA libraries described also confirms that all the α and β chain transcripts are less than the full length of 1.3 kb for α and 1.0 kb for β (Y.Y., unpublished observations). These truncated transcripts are presumably composed of only *D-J-C* or *J-C* sequences²¹⁻²³. Thus the decrease in α - and β -chain transcripts in *nu/nu* mice is not solely due to the lower numbers of Thy-1⁺ cells.

A different and interesting picture is seen for γ -chain messages. Figure 2 clearly shows that after nylon-wool enrichment more γ -chain message can be found. T cells from athymic mice express a several times higher level of γ -chain messages. The majority of the γ cDNAs are 1.6 kb and are presumably 'full length'.



Thy-1⁺ T cells from athymic mice (purified using nylon wool) also contain a reduced level of both the α and β gene transcripts. This may be due to the presence of a different subpopulations of Thy-1⁺ T cells in athymic spleens, possibly those of less mature T cells when compared to the normal mice. The finding that the α and β transcripts that do exist in young athymic mice are truncated is consistent with such a hypothesis. Another possibility is that the lower level of α and β TcR transcripts is due to a different environment in the athymic mice spleen or the predominance of a different subset(s) of splenic T cells in these *nu/nu* mice.

It is interesting that although the levels of α - and β -chain TcR transcripts in athymic mice are dramatically reduced, the γ level in these mutant mice is elevated. The reason for this is not yet known. It is possible that the rearrangement and expression of γ -chain message occur before exposure to the thymus or is independent of the thymic environment, so that

Double-stranded (ds) cDNA was synthesized from total RNA derived from spleens of *nu/+* (8 weeks) and *nu/nu* (8 weeks) according to a modified procedure of Gubler and Hoffman³⁰. After addition of the *Eco*RI linker, the cDNAs were cloned into λ gt10. About 10^6 independent recombinant phages were plated on *E. coli* (C600 hf) without amplification and screened using nick-translated constant probes of α , β and γ chain cDNA.

processing of these genes can occur outside the thymus. Although the study of the ontogeny of the expression and rearrangement of the T-cell genes suggests an order of γ , β then α ^{9,10}, these studies were done exclusively in the context of thymic environment. Moreover, rearrangement of γ -chain genes before β -chain genes outside the thymus or in fetal thymus has not been documented. It is possible that the rearrangement and expression of 'functional' γ -chain messages is more efficient than those of the α and β chain genes in the extrathymic pathways of T-cell differentiation in athymic mice.

Perhaps the most exciting observation is that athymic mice contain a population(s) of T cells in which the γ -chain T-cell receptors are used. It has been suggested that γ -chain gene products may be involved in the recognition of class I MHC gene products in a 'two receptor' model (one for antigen and one for MHC gene products)⁹, but several recent findings suggest that the situation may be more complicated. First, although it was thought that γ -chain genes are limited to cytotoxic T cells²⁶, helper T cells (especially autoreactive clones) have also been found to contain these messages²⁷. Secondly, Dembic *et al.*²⁸ have reported the successful reconstitution of a new T cell specificity recognizing the antigen fluorescein in the context of murine class I D^d products by DNA transfer of only the α - and β -chain genes from a T cell with the same specificity. This result suggests that the α - β heterodimer is sufficient for the recognition of both antigen and MHC gene products. Finally, with only one exception, over thirty γ transcripts isolated from cytotoxic T lymphocytes^{16,17,34}, mature T lymphocytes and thymocytes¹⁸ contain deletions, frameshift mutations, or unspliced messages. Thus, it is likely that γ gene products may be important in a subpopulation or lineage of T cells responsible for a second type of immunorecognition that has yet to be identified. Our observation that all four cDNAs obtained from athymic spleen cells are functional is in sharp contrast to the existing data on γ messages from functional T cells and thymocytes^{16-18,34}. The finding of no detectable full length α - and β -chain transcripts in young athymic mice suggests that the majority of these cells may not have the α - β heterodimer on their surfaces. Athymic spleen cells should thus be the ideal cells for the study of the possible role of γ gene product. They may contain populations of either pre-T cells or T cells in an extrathymic pathway, which may be involved in the establishment of tolerance or the recognition of self MHC gene products. Other possibilities include γ gene products being receptors active in primitive defense mechanisms or receptors that recognize antigens and MHC gene products.

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Note added in proof: A decrease of α -chain and β -chain messages in athymic mice³⁵ and the detection of γ -chain protein on the surface of human T cells have been reported³⁶⁻³⁸.

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Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance

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Increased expression of P-glycoprotein, a plasma membrane glycoprotein of relative molecular mass (M_r) 170,000 (170K), occurs in a wide variety of cell lines that exhibit pleiotropic resistance to unrelated drugs¹⁻⁴. The presence of P-glycoprotein in human cancers refractory to chemotherapy suggests that tumour cells with multidrug resistance can arise during malignant progression⁵. We have discovered striking homology between P-glycoprotein and the HlyB protein, a 66K *Escherichia coli* membrane protein required for the export of haemolysin (protein of M_r 107K). P-glycoprotein can be viewed as a tandem duplication of the HlyB protein. The hydropathy profiles of the two proteins are similar and reveal an extensive transmembrane region resembling those found in pore-forming plasma membrane proteins. The C-terminal region of P-glycoprotein and the HlyB protein contain sequences homologous to the nucleotide-binding domains of a group of closely related bacterial ATP-binding proteins. We propose a model for multidrug resistance in which P-glycoprotein functions as an energy-dependent export pump to reduce intracellular levels of anticancer drugs.

A search of the GenBank DNA Sequence Database (using the FASTN program from the National Biomedical Research Foundation (NBRF) Protein Identification Resource) detected extensive homology between the 3' end of a P-glycoprotein complementary DNA clone (pL20, see Fig. 1 legend) and the *hlyB* gene of *E. coli*. This gene is one of four involved in the production and transport of haemolysin: *hlyA* encodes the α haemolysin molecule (107K), *hlyC* encodes a small (20K) cytoplasmic protein that may be involved in activating haemolysin, whereas *hlyB* and *hlyD* encode membrane proteins required for the export of α haemolysin from the bacterium⁶ (for reviews see refs 7-10).

When the deduced amino-acid sequences of the *hlyB* gene⁶ and pL20 are compared graphically, extensive C-terminal homology is evident (Fig. 1a). Whereas there is little direct

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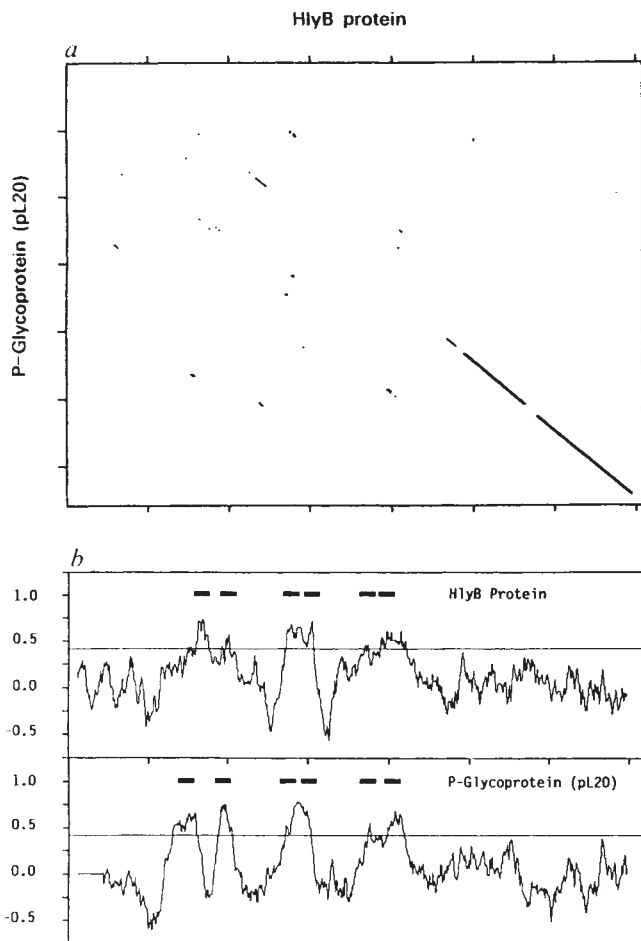


Fig. 1 Comparison of P-glycoprotein (C-terminal 655 amino acids) and the HlyB protein (707 amino acids). *a*, Segment comparison plot generated by the DOTMATRIX program from the National Biomedical Research Foundation (NBRF) Protein Identification Resource using the mutation probability data matrix for amino-acid replacements for scoring³⁵. The window size was 21 amino acids and the minimum score required to generate a point was 30. Each division on the axes corresponds to 100 amino acids. *b*, Hydropathy profiles of the HlyB protein (top) and P-glycoprotein (bottom) generated using the normalized hydrophobicity values of Eisenberg *et al.*¹¹ and a window size of 21 amino acids. Regions above the line at 0.42 are candidates for membrane-spanning segments. The six predicted transmembrane segments in each sequence, as identified using the criteria of Eisenberg *et al.*¹¹, are indicated by solid bars. The mean hydrophobicity for 21 amino acid segments is indicated on the vertical axis. Divisions on the horizontal axis correspond to 100 amino acids.

Methods. The pCHP1 cDNA probe³¹ was used to select a 2,321-base pair (bp) cDNA clone (pL20) from an Okayama and Berg pcD vector-library prepared from a drug-sensitive Chinese hamster ovary cell line as described³⁶. For DNA sequencing, pL20 DNA was digested with *Pst*I or *Bam*HI and cloned into M13mp9. Both strands were sequenced using synthetic oligonucleotide primers and the dideoxy method³⁷. The pL20 clone contains a 1,965-bp open reading frame followed by a 355-bp untranslated region and a poly(A) tail (sequence available on request). The 640-bp sequence from positions 1,167 to 1,806 is homologous to pCHP1.

homology in the remainder of the amino-acid sequence, hydropathy profiles of the two sequences are remarkably similar (Fig. 1b). Both proteins have a large hydrophobic region containing six potential transmembrane segments arranged in pairs. The hydrophobicities and the hydrophobic moments^{11,12} of these putative transmembrane segments are consistent with channel-forming functions. The C-terminal region of P-glycoprotein has been localized to the cytoplasmic side of the plasma membrane with monoclonal antibodies³. Consequently, the hydropathy plots indicate that only the short stretches of amino acids joining paired transmembrane segments are exposed on the cell surface.

Sequence data from overlapping cDNA clones¹³ indicate that the P-glycoprotein gene consists of a tandem duplication (unpublished observations), a structure confirmed in a functional, full-length mouse cDNA clone (P. Gros, personal communication). This, coupled with the protein structural analysis (Fig. 1), implies that P-glycoprotein is a near-perfect tandem duplication of the HlyB protein.

Only three breaks in the P-glycoprotein C-terminal domain are required to produce an excellent alignment with the 230 C-terminal amino acids of the HlyB protein (Fig. 2). Of the 228 possible amino acid matches, 107 (46.9%) are identical and a further 63 (27.6%) are conserved substitutions. This suggests these two proteins share both a common function and a common history. A search of the NBRF Protein Sequence Database (using the FASTP program) identified two other bacterial transport proteins, the HisP protein of the histidine permease system of *Salmonella typhimurium*¹⁴ and the MalK protein of the maltose and maltodextrin transport system of *E. coli*¹⁵, with significant homology with this region. These two proteins have homology with each other¹⁶ as well as with the PstB protein of the phos-

phate-specific transport system of *E. coli*¹⁷ and the OppD protein of the oligopeptide permease of *S. typhimurium*¹⁸. These four proteins are membrane-associated components of high-affinity, substrate-binding dependent, active transport systems (for a review see ref. 19). Homology between P-glycoprotein and these proteins is greatest in two regions (indicated A-A and B-B in Fig. 2) that are involved in nucleotide (probably ATP) binding and are thought to energize transport directly¹⁸.

The extent of the protein sequence homology between P-glycoprotein, the HlyB protein, the HisP/MalK/PstB/OppD proteins and five other nucleotide-binding proteins is shown by cluster analysis (Fig. 3). The highest homology occurred between P-glycoprotein and the HlyB protein. Furthermore, the HisP/MalK/OppD cluster is more similar to the P-glycoprotein/HlyB cluster than to the bacterial OppD protein. This suggests both P-glycoprotein and the HlyB protein couple energy production to transport in a manner analogous to the binding-protein dependent bacterial transport systems. Although the cluster analysis does not give direct evolutionary relationships, the high degree of amino-acid sequence homology suggests that these proteins arose from a common ancestor and homology has been maintained by selection pressure resulting from functional similarities in these transport systems. Such a degree of evolutionary conservation between a eukaryotic protein (excluding mitochondrial and chloroplast proteins) and a bacterial protein is unprecedented, and raises the question of ancestry. Based on codon usage, it has been suggested⁶ that *E. coli* has only recently acquired the chromosomal haemolysin gene group from a distantly related organism. It is therefore possible that *E. coli* has acquired at least part of the *hlyB* gene from a mammalian P-glycoprotein gene.

ARG	PRO	ASP	ILE	PRO	VAL	---	LEU	GLN	GLY	LEU	SER	LEU	GLU	VAL	LYS	LYS	GLY	GLN	THR	LEU	ALA	LEU	VAL	GLY	SER	SER	GLY	CYS	GLY	452	
***	***	***	***	***	***	---	***	***	---	***	***	---	---	***	***	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	507
LYS	PRO	ASP	SER	PRO	VAL	ILE	LEU	ASP	ASN	ILE	ASN	LEU	SER	ILE	LYS	GLN	GLY	GLU	VAL	ILE	GLY	ILE	VAL	GLY	ARG	SER	GLY	SER	GLY		
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LYS	SER	THR	LEU	THR	LYS	LEU	ILE	GLN	ARG	PHE	TYR	ILE	PRO	GLU	ASN	GLY	GLN	VAL	LEU	ILE	ASP	GLY	HIS	ASP	LEU	ALA	LEU	ALA	ASP		
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PRO	ASN	TRP	LEU	ARG	ARG	GLN	VAL	GLY	VAL	VAL	LEU	GLN	ASP	ASN	VAL	LEU	LEU	ASN	ARG	SER	ILE	ILE	ASP	ASN	ILE	SER	LEU	ALA	ASN		
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PRO	GLY	MET	SER	VAL	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		
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ASN	THR	ARG	VAL	GLY	ASP	LYS	GLY	THR	GLN	LEU	SER	GLY	GLY	GLN	LYS	GLN	ARG	ILE	ALA	ILE	ALA	ARG	ALA	LEU	VAL	ARG	GLN	PRO	HIS	572	
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ASN	THR	ILE	VAL	GLY	GLU	GLN	GLY	ALA	GLY	LEU	SER	GLY	GLY	GLN	ARG	GLN	ARG	ILE	ALA	ILE	ALA	ARG	ALA	LEU	VAL	ASN	ASN	PRO	LYS		
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ILE	LEU	LEU	LEU	ASP	GLU	ALA	THR	SER	ALA	LEU	ASP	THR	GLU	SER	GLU	LYS	VAL	VAL	GLN	GLU	ALA	LEU	ASP	LYS	ALA	ARG	GLU	GLY	ARG	602	
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ILE	LEU	ILE	PHE	ASP	GLU	ALA	THR	SER	ALA	LEU	ASP	TYR	GLU	SER	GLU	HIS	VAL	ILE	MET	ARG	ASN	MET	HIS	LYS	ILE	CYS	LYS	GLY	ARG		
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THR	CYS	ILE	VAL	ILE	ALA	HIS	ARG	LEU	SER	THR	ILE	GLN	ASN	ALA	ASP	LEU	ILE	VAL	VAL	ILE	GLN	ASN	GLY	LYS	VAL	LYS	GLU	HIS	GLY	632	
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THR	VAL	ILE	ILE	ILE	ALA	HIS	ARG	LEU	SER	THR	VAL	LYS	ASN	ALA	ASP	ARG	ILE	ILE	VAL	MET	GLU	LYS	GLY	LYS	ILE	VAL	GLU	GLN	GLY		
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THR	HIS	GLN	GLN	LEU	LEU	ALA	GLN	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	655	
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	707	
LYS	HIS	LYS	GLU	LEU	LEU	SER	GLU	PRO	GLU	SER	LEU	TYR	SER	TYR	LEU	TYR	GLN	LEU	GLN	SER	ASP										

Fig. 2 Aligned sequences of the C-terminal 232 amino acids of P-glycoprotein (upper sequence) and the C-terminal 230 amino acids of the HlyB protein (lower sequence). The alignment was generated by the ALIGN program from the NBRF Protein Identification Resource using the mutation probability data matrix for amino-acid replacements with a bias of two and a break penalty of six for scoring³⁵. Amino acids identical in both sequences are indicated by ***. Conserved amino-acid substitutions (scores greater than zero in the mutation probability data matrix for amino-acid replacements³⁵) are boxed. The numbers at the ends of lines indicate the position in the sequence of the last amino acid in that line. Three gaps introduced for alignment (single amino-acid breaks following positions 429 and 640 in the pL20 sequence and a two amino-acid break at position 572 in the HlyB sequence) are indicated (---). The underlined regions (A-A and B-B) contain the putative nucleotide-binding site and are highly conserved in other bacterial nucleotide-binding proteins (see Fig. 3). Region A-A, characterized by the sequence hhG1/mSG(C/S)GKST ('h' indicates a hydrophobic amino acid conserved in P-glycoprotein, the HlyB protein and the HisP/MaK/PstB/OppD proteins), is glycine-rich and capable of forming a flexible loop that may undergo conformational change either on substrate binding or because of interaction with another protein domain³⁸. Region B-B contains four consecutive hydrophobic amino acids (which probably exclude water from the bound nucleotide to reduce hydrolysis³⁸) followed by two acidic amino acids (hhhhDE). The high homology between P-glycoprotein and the bacterial transport proteins extends over 32 amino acids in this region suggesting an additional function(s). As this is the most conserved amino-acid sequence among the four binding-protein dependent transport proteins, it may couple energy production directly to transport.

Based on homology and structural similarities between P-glycoprotein and the HlyB protein and on the homology with the four bacterial binding-protein dependent transport proteins, we propose that P-glycoprotein functions in the plasma membrane to export molecules from the cell. It couples energy in the form of ATP directly to transport and effects transport through a channel formed by the transmembrane segments (Fig. 4).

P-glycoprotein may export drugs directly (Fig. 4a). This would require that the protein should not only be able to bind specifically many structurally diverse drugs but also that it should be able to release these drugs to the extracellular medium. Support for such binding ability is provided by the vinblastine photoaffinity labelling of a 170K glycoprotein (P-glycoprotein?) in membrane vesicles from multidrug-resistant cells^{20,21}. The requirement for P-glycoprotein to recognize these compounds is eliminated if P-glycoprotein exports a protein rather than drugs (Fig. 4b). Drugs bound by this protein (not necessarily reversibly) would be removed when the protein was exported. Such binding might occur through hydrophobic domains such as those present in α haemolysin⁶. We are examining culture medium from multidrug-resistant cells for such a protein.

If P-glycoprotein functions in multidrug resistance in a manner analogous to that of the HlyB protein in haemolysin export, it is reasonable to expect these processes to have common aspects. When inhibitors of energy production (for example potassium cyanide, 2,4-dinitrophenol, sodium azide) are added to mammalian cells in the absence of energy sources such as glucose, the net accumulation of drug is increased²²⁻²⁶. This effect is greater in multidrug-resistant cells and, when glucose is restored, the intracellular concentrations of drug decrease more rapidly than in drug-sensitive cells. An energy-dependent, efflux pump for drugs has been suggested as the mechanism of multidrug resistance^{22,25,27}. These compounds also inhibit the transport of haemolysin²⁸. Additionally, local anaesthetics (such as procaine), reduce the level of resistance of multidrug-resistant cells^{29,30} and inhibit the export of haemolysin²⁸.

These findings have implications for cancer chemotherapy. Although the homology reported here is for Chinese hamster P-glycoprotein, the conservation of P-glycoprotein, as judged by monoclonal antibodies³ and DNA hybridization³¹, makes it virtually certain that this homology will extend to other mammalian (including human) P-glycoprotein molecules. It may be possible to screen for compounds to overcome multidrug

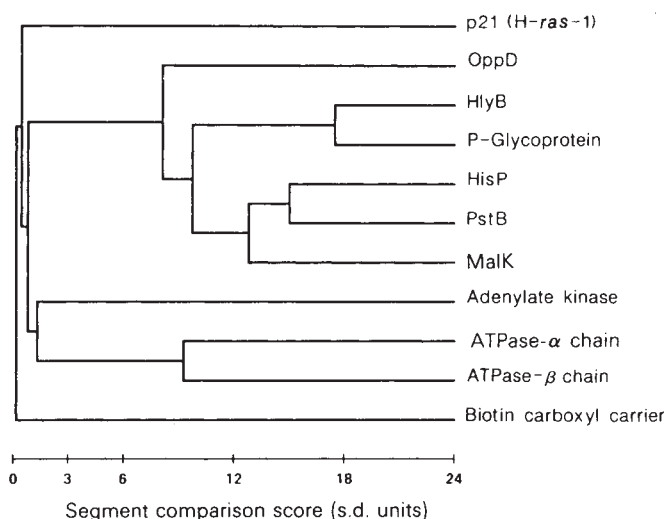


Fig. 3 Cluster analysis of the protein sequence homology of some nucleotide-binding proteins. The RELATE program from the NBRF Protein Identification Resource³⁵ was used to determine objectively similarities between P-glycoprotein (pL20), the HlyB protein and nine proteins with nucleotide-binding regions homologous to regions A-A and B-B in Fig. 2. Besides the HisP, MalK, PstB and OppD proteins, adenylate kinase (rabbit), the H-ras-1 p21 transforming protein (human), the biotin carboxyl carrier protein from *Propionibacterium shermanii* and the α and β chains of the *E. coli* F_1F_0 -ATPase^{38,39} were examined. These proteins were selected from known nucleotide-binding proteins because each has both of the nucleotide-binding regions present in the bacterial transport proteins and because they represent a functionally diverse group³⁸. The amino-acid sequences were from the NBRF Protein Sequence Database. For each pair of proteins, the homology scores between all possible 25 amino-acid segments of one protein with all possible 25 amino acid segments in the other protein were determined using the mutation probability data matrix for amino-acid replacements³⁵. A numerical value derived from the distribution of scores was then determined for the actual sequences of the two proteins and for 500 runs performed using computer-generated randomized sequences of both proteins. The difference between the value for the actual sequences and the mean value for the randomized runs was then divided by the standard deviation of the values from the randomized runs to yield a segment comparison score with units of standard deviation (s.d.). The probability of obtaining such a score by chance can then be estimated from a cumulative standardized normal distribution table. This analysis has the advantage of being relatively insensitive to differences in the lengths of the proteins being compared³⁵. Clusters were formed by merging proteins with the highest segment comparison scores. As clusters were formed, the score used for merging was the weighted average of the scores of the members of a cluster. Note that cluster analysis forces the eventual merging of all proteins. Only statistically significant groups should be considered representative of structural homology. Thus we consider only the group consisting of P-glycoprotein, the HlyB protein and the HisP/MalK/PstB/OppD proteins and the group containing the ATPase α and β chains to be significant (the probability of obtaining a score >6 s.d. is $<10^{-9}$ whereas the probability of a score >10 s.d. is $<10^{-23}$).

resistance based on their ability to inhibit haemolysis export. In a similar manner, it may be possible to screen existing and potential anticancer agents for their differential ability to kill haemolytic and non-haemolytic bacteria. In this context, it is intriguing that there is a brief report that a nalidixic acid-resistant strain of *E. coli* had also become haemolytic³².

Although we have presented a model for the role of P-glycoprotein overexpression in multidrug resistance, the function of P-glycoprotein in drug-sensitive cells is not clear. It may serve to protect cells from low levels of toxic lipophilic compounds and its role in multidrug resistance could simply be an

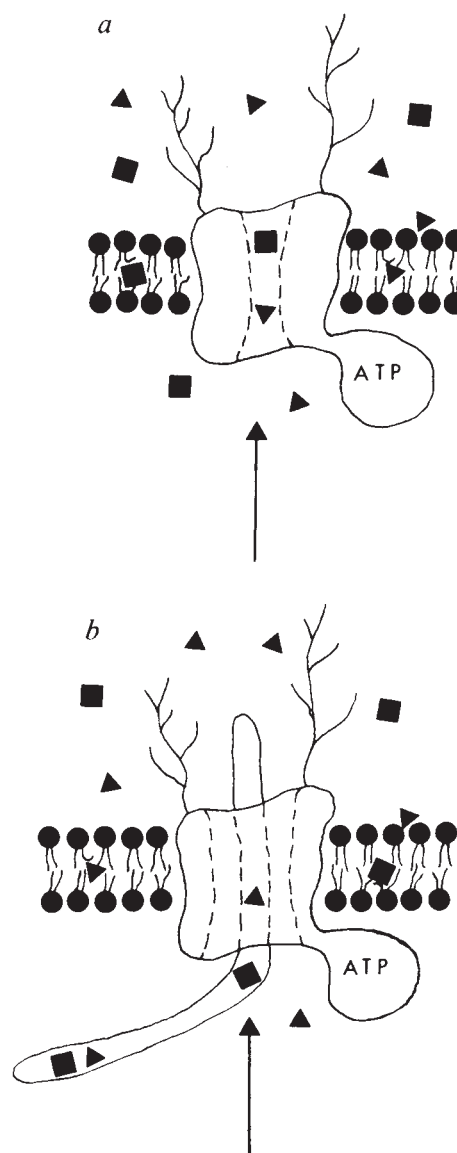


Fig. 4 Models for the role of P-glycoprotein in multidrug resistance. In *a* P-glycoprotein exports two structurally different drugs (■, ▲) directly through a channel. In *b* the drugs are bound by an unidentified protein that is then exported from the cell by P-glycoprotein. The presumptive ATP-binding site (ATP) is on the cytoplasmic side of the plasma membrane. Drugs are shown entering the cell by diffusion through the lipid bilayer. Arrows indicate the direction of movement through the membrane channel. For clarity, only half the P-glycoprotein tandem duplication is shown. In keeping with known channel-forming proteins¹², several P-glycoprotein molecules may be required to form this channel.

extension of this function in response to increased concentrations of such compounds. Alternatively, P-glycoprotein may have a transport function unrelated to multidrug resistance and only upon gross exaggeration of this function (for example by gene amplification) does resistance become apparent. If this is the case, then the most likely candidates for transport by P-glycoprotein are, by analogy with haemolysin transport, large proteins. One group of potentially haemolysin-like proteins is the pore-forming molecules released by cytotoxic cells such as eosinophils, cytotoxic T lymphocytes and NK-like cells^{33,34}. These molecules appear to be well conserved, in keeping with the conserved nature of P-glycoprotein.

The potential existence of a previously unknown protein-translocation mechanism in eukaryotic cells is perhaps the most important aspect of the homology between P-glycoprotein and the HlyB protein. The high degree of conservation of P-glycoprotein suggests this protein plays a fundamental role in mammalian cells. As P-glycoprotein appears to be encoded by a multigene family³¹, there may be a number of homologous systems, each translocating a different protein or proteins.

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Note added in proof: The homology between the binding-protein dependent transport proteins and the HlyB protein has been independently reported⁴⁰. The nucleotide-binding region of these proteins also has homology with bacterial proteins involved in cell division⁴⁰, nodulation during nitrogen fixation⁴⁰ and DNA repair⁴¹.

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Mature murine B lymphocytes immortalized by Kirsten sarcoma virus

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Clonal, antigen-specific, functionally responsive cell populations have proved critical for the analysis of the activation and regulation of lymphocytes. Such studies with B lymphocytes, the precursors of antibody-secreting cells, are hampered by the difficulty in generating phenotypically mature, antigen-reactive lines from defined cell populations. One method is to use acutely transforming retroviruses, which can transform B-lineage lymphocytes *in vitro*. However, Abelson murine leukaemia virus (A-MuLV) infection of murine bone marrow cells *in vitro* yields mostly immature B-cell lines¹⁻⁵, and infection of murine bone marrow cells with murine sarcoma viruses carrying *ras* related genes produces only immature lymphoid cell lines⁶. Retroviruses which contain *ras* can immortalize nonlymphoid cells without causing loss of mature phenotypic characteristics⁷. We used *ras*-containing Kirsten sarcoma virus (KiSV) pseudotyped with an amphotropic MuLV helper virus, to infect a purified population of mature, hapten-binding murine splenic B lymphocytes, aiming to generate mature B-cell lines to use as models for the study of B-cell growth and differentiation physiology. Immortalized B-cell lines which retain the same mature phenotype as the starting population, including hapten-specific binding, were produced. This is the first demonstration of a method for immortalizing selected antigen-binding B lymphocytes, and the first example of immortalization of mature B cells *in vitro* with an acutely transforming *ras*-containing retrovirus.

In most reported studies of retroviral transformation of B-lineage lymphocytes *in vitro*, the starting cell populations are heterogeneous, immature haematopoietic precursors. With

suitable manipulation of culture conditions, surface immunoglobulin positive lines are obtained³. A-MuLV infection of clonal pre-B cells gives rise to B-cell lines which express surface immunoglobulin⁴. These transformed lines are of unknown antigen specificity and are often autonomously growing and tumorigenic, or depend on nonphysiological stimuli such as high concentrations of lipopolysaccharide for continued growth⁵. We infected mature B lymphocytes, selected on the basis of antigen-specific receptor expression, with an acutely transforming retrovirus. To maximize the efficiency of infection of such phenotypically differentiated cells, we used viral pseudotypes composed of the transforming retrovirus and an amphotropic helper virus. We isolated 2,4,6-trinitrophenol (TNP)-binding B cells (TNP-B cells) from the spleens of unimmunized (C3H × DBA/2)F₁ (C3D2F₁) mice by binding to and elution from TNP-gelatin plates. This method normally enriches TNP-specific plaque-forming cells 50-100-fold and 50-90% of the cells form rosettes with TNP-modified sheep red blood cells⁸. KiSV pseudotyped with amphotropic (virus 4070) or ecotropic (Moloney MuLV) helper virus was propagated in BALB/c 3T3 cells (BALB/3T3), as previously described⁷. We used an amphotropic helper virus because acute transforming viruses tend to infect and immortalize only immature stem cells because of a limitation of target cell host range and this may be overcome using a helper virus with a broad range of cell/tissue infectivity^{7,9,10}. TNP-B cells were cocultivated with lethally irradiated BALB/3T3 cells producing amphotropic pseudotyped KiSV (KiSV-ampho) in microwell culture plates. Growing colonies of lymphoblastoid cells were found in 6-25% of wells in 1-3 weeks (Table 1). Between 12-26% of wells gave rise to colonies in different experiments, consistent with each colony being clonal in origin by Poisson statistical analysis. Assuming that the colonies of proliferating cells in each well were clonal, the efficiency of outgrowth of colonies was from 3×10^{-6} to 1.2×10^{-5} . Long-term cell lines were established from these colonies and have been growing continuously for 13 months. The characteristics of two of these lines are described below. No lymphoblastoid colonies were observed in cocultivations of TNP-B cells with BALB/3T3 cells producing amphi-MuLV

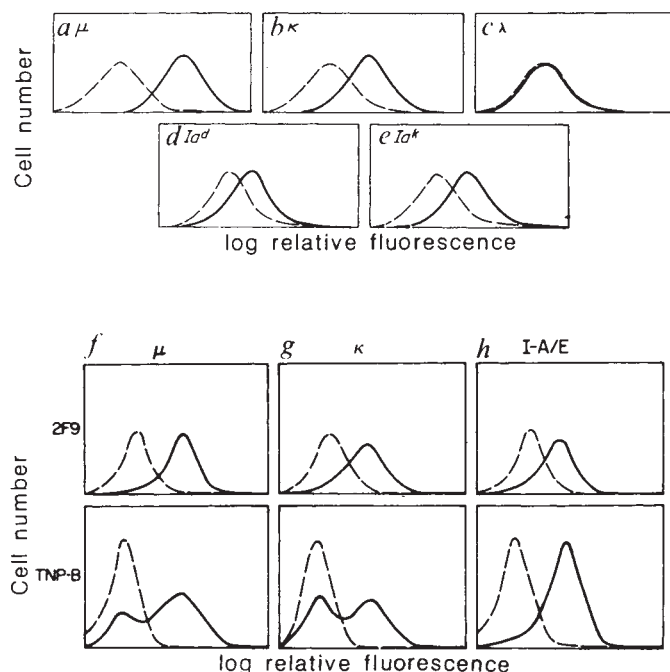


Fig. 1 Surface immunoglobulin and Ia expression on a KiSV-immortalized B-cell line. *a-e*, 2F9 cells were incubated with hybridoma supernatants specific for: *a*, IgM (Bet 2) (ref. 12); *b*, Ig κ light chain (187.1) (ref. 13); *c*, Ig λ light chain (4/101) (from T. Imanishi-Kari, MIT); *d*, I-A^d (MKD6) (ref. 14); *e*, or I-A^k (10.2.16) (ref. 15), for 30 min at 0 °C, followed by fluorescein-conjugated mouse anti-rat κ (FITC-MAR 18.5, Becton-Dickinson) (*a-c*), or fluorescein-conjugated goat anti-mouse IgG (*d, e*) for 30 min at 0 °C; *f-h*, 2F9 and freshly isolated C3D2F₁ TNP-binding B cells were incubated with Bet 2, 187.1 or M5/114 (rat anti-mouse I-A/E) hybridoma supernatants, followed by FITC-MAR 18.5. Stained cells were fixed in 1% paraformaldehyde and examined by flow cytometry (solid traces). Dotted traces, nonspecific fluorescence (identical samples incubated with fluorescein-conjugated reagents alone). Note that 2F9 and TNP-B cells show comparable intensities of fluorescence, but the 2F9 cells are significantly larger (average diameter 12.5 μ m compared to 6.4 μ m for normal B cells) and, therefore, express IgM, κ and I-A/E at significantly lower densities. Also, approximately 65% of the TNP-B cells are IgM, κ ⁺ (but 100% are Ia⁺)—this may be due to immunoglobulin modulation during affinity purification or presence of Ia⁺ non-B cells in this population.

alone, or with uninfected BALB/3T3 cells. TNP-B cells were also cocultivated with BALB/3T3 cells producing KiSV with Moloney-MuLV helper virus. The titres of sarcoma virus produced by KiSV (ampho)- or KiSV (Moloney)-infected BALB/3T3 cells were similar, at approximately 5×10^7 focus forming units per ml. In the cultures with KiSV (Moloney) 10% of wells showed colony growth, but no lines could be established from such colonies. No viable cells could be recovered from cultures of TNP-B cells alone after 1 week.

Two cell lines derived from KiSV infection of TNP-B cells (2F9 and 6H3) have been extensively characterized. These lines have doubling times of 24 and 6–12 h, respectively, and grow as nonadherent cells in liquid culture medium with 10 or 20% fetal bovine serum and 2.5×10^{-5} M 2-mercaptoethanol. The phenotype of these cells was analysed by fluorescence-flow cytometry. Both 2F9 and 6H3 express surface IgM with κ light chain (Fig. 1*a*). In addition, they both express Ia determinants of the H-2^k and H-2^d haplotypes (Fig. 1*a*). Compared to purified C3D2F₁ TNP-binding B cells, the KiSV-immortalized lines express lower densities of surface IgM and I-A/E determinants (Fig. 1*b*).

The hapten specificity of 2F9 and 6H3 has been analysed by two methods. First, up to 25% of these cells form rosettes with

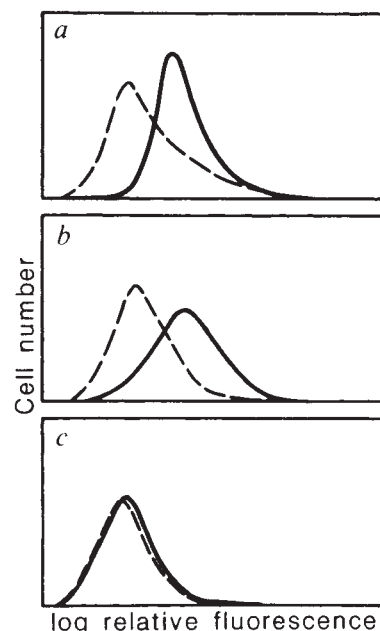


Fig. 2 TNP-HSA binding to KiSV-immortalized B-cell lines. Cells from the KiSV-immortalized B-cell lines 2F9 and 6H3 (*a, b*), and *c*, the murine B-cell lymphoma (ref. 8) were incubated with: dotted line, 20 μ g ml⁻¹ human serum albumin (HSA); solid line, TNP-conjugated HSA (TNP-HSA), for 30 min at 0 °C, followed by fluorescein-conjugated goat anti-HSA for 30 min, 0 °C. Paraformaldehyde-fixed cells were then analysed by flow cytometry.

TNP-modified sheep erythrocytes (TNP-SRBC). Such rosette assays depend on the amount of hapten bound to the SRBC and the avidity of the surface receptors for the hapten (A.K.A., unpublished data). It is therefore not surprising that only a fraction of the cells formed rosettes. No rosettes are seen in suspensions of these KiSV-immortalized B cells with unmodified sheep red blood cells, nor in suspensions of non-TNP-specific lines (such as the murine B-cell lymphoma A20) and TNP-SRBC (data not shown). The specificity of rosette formation was confirmed in inhibition assays using 2,4-dinitrophenyl (DNP)-lysine. Inhibition of rosetting is 50% at a concentration of 6×10^{-6} M DNP-lysine with normal C3D2F₁ TNP-B cells and at 1.6×10^{-5} M with the 2F9 line. This suggests that 2F9 is an immortalized B cell whose receptors are of relatively low affinity; the population of normal hapten-binding B lymphocytes is heterogeneous. Hapten binding was also analysed by flow cytometry. Both 2F9 and 6H3 preferentially bind TNP-modified human serum albumin (TNP-HSA) over unmodified HSA, while a control BALB/c B lymphoma (A20.2J) that is not specific for TNP, shows no such preference (Fig. 2). Thus the hapten specificity of the original uninfected B cells is retained in the immortalized lines.

Viral gene expression in the KiSV-immortalized B-cell lines was assayed by probing for viral transcripts. Cytoplasmic RNA prepared from these cell lines was analysed for the presence of Ki-ras homologous RNA by dot blot hybridization using a *v-ras* probe derived from KiSV. Significant levels were found in both 2F9 and 6H3 (Fig. 3). Low or undetectable levels of *v-ras* RNA were present in other murine lymphoblastoid lines (for instance A20.2J) and in normal splenic TNP-B cells (Fig. 3). Cytoplasmic RNA dot blot hybridizations using an amphotropic-MuLV *env* specific probe⁷ also indicated a high level of MuLV-specific RNA in 2F9 and 6H3 (not shown). Thus, transcripts from both the helper virus and the sarcoma virus are present in the KiSV-immortalized lines. Pelletable reverse transcriptase activity was measured in ultracentrifuged culture supernatants, by a standard technique in which [³²P]dTTP is incorporated into DNA using

Table 1 Generation of lymphoblastoid colonies by cocultivation of murine splenic B cells with virus-producing BALB/3T3 cells

Splenic B-cell target population	Virus (helper)	Positive wells	Estimated efficiency
TNP-binding (C3H × DBA/2)F ₁	KiSV (ampho-MuLV)	43/250	0.86×10^{-5}
TNP-binding (C3H × DBA/2)F ₁	KiSV (Moloney-MuLV)	5/50	0.5×10^{-5}
TNP-binding (C3H × DBA/2)F ₁	Ampho-MuLV	0/50	0
TNP-binding (C3H × DBA/2)F ₁	None	0/200	0

TNP-binding spleen cells were purified from (C3H × DBA/2)F₁ (C3D2F₁) mice by binding to and elution from TNP-gelatin plates. These cells were mixed with γ -irradiated BALB/3T3 cells (10,000 rad) producing Kirsten sarcoma virus pseudotyped with either amphotropic or ecotropic (Moloney) murine leukaemia virus in RPMI 1640 supplemented with 20% fetal bovine serum, 2.0×10^{-5} M 2-mercaptoethanol and $5 \mu\text{g ml}^{-1}$ polybrene (Sigma). B cells (2×10^{-4}) were plated with 5×10^{-4} BALB/3T3 cells in 200 μl flat bottom microculture wells. Cultures were fed with fresh medium every 2–4 days. Wells were microscopically assayed for the presence of growing cells. The number of microwells with growing lymphoblastoid clusters appearing within 1–6 weeks (positive wells) and the fraction of plated B cells induced to grow out into colonies, assuming a clonal origin of these colonies (estimated efficiency), are given. Control cultures were plated with either no BALB/3T3 cells, or BALB/3T3 cells producing amphotropic MuLV alone. The data are pooled from three separate experiments.

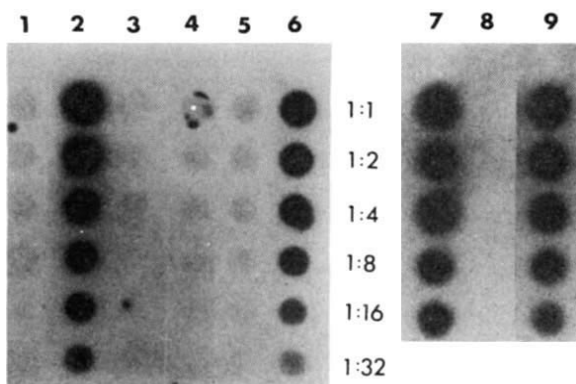


Fig. 3 RNA dot blot analysis of B-cell lines for Ki-ras specific RNA. Ki-ras homologous cytoplasmic RNA was analysed in: lanes 1 and 8, Abelson-MuLV-immortalized B-cell lines 1D12 and 12E11 (respectively); lanes 2 and 7, KiSV-immortalized B-cell lines 2F9 and 6H3 (respectively); lane 3, normal murine splenic B cells; lane 4, the murine B-cell lymphoma A20.2J; lane 5, Abelson-MuLV (amphotropic MuLV)-producing BALB/3T3 cells; lanes 6 and 9, KiSV (amphotropic-MuLV)-producing BALB/3T3 cells. Cytoplasmic RNA was prepared from 4×10^6 cells of each type by NP-40 lysis and denatured in 5% formaldehyde at 55°C for 15 min. Samples were serially 2-fold diluted and blotted onto nitrocellulose with a vacuum manifold (Schleicher & Schuell). Blots were baked at 80°C for 1 h under vacuum and hybridized to a ^{32}P -labelled Ki-ras specific probe (the 600 base-pair *Sst*II/*Hind*II fragment from KiSV) (ref. 7) in 10% dextran, $5 \times \text{SSC}$, at 42°C , for 18 h. The final washes of the blots were in $0.2 \times \text{SSC}$ at 50°C . Autoradiography was carried out at -80°C for 18 h using intensifying screens^{7,16}.

a poly(A) template and trichloroacetic acid precipitable radioactivity is measured¹⁷. Supernatant from cultures of 2F9 (10 ml) yielded 8,110 counts per minute (c.p.m.) in this assay, compared to 677 c.p.m. from an uninfected BALB/3T3 culture supernatant, and 128,761 c.p.m. from the culture supernatant of a Mo-MuLV-producing BALB/3T3 line. Thus 2F9 produces low levels of viral particles.

We have shown here that introduction of the *v-ras* oncogene into mature B lymphocytes by retroviral infection can immortalize them. This is the first demonstration of *ras*-induced immortalization of mature lymphocytes *in vitro* and it is likely that the use of an amphotropic helper virus was important to our success. KiSV pseudotyped with an ecotropic helper (Moloney-MuLV) generated a few lymphoblastoid colonies none of which could be established as permanent lines (Table 1). The amphotropic envelope allows infection of cells that do not express membrane receptors for ecotropic viral envelope glycoproteins^{9,10}. The same approach has been used to immortalize differentiated human umbilical vein endothelial cells⁷, and to infect mature

helper T-cell lines *in vitro*¹¹. Mature B cells may have few or no functional receptors for ecotropic retroviruses, but do have amphotropic retroviral receptors.

The cell lines described here retain their mature phenotype, and preliminary functional studies indicate that they can be further mitotically stimulated by reagents which stimulate normal B cells, particularly the helper T-cell-derived lymphokine, B-cell stimulatory factor 1 (data not shown). These cells do not secrete immunoglobulin constitutively; we are attempting to induce increases in immunoglobulin gene transcription, biosynthesis and/or secretion with activating stimuli, including lymphokines. The technique of infecting purified, functionally well-defined lymphocyte populations with acute transforming retroviruses will be useful for the development of model systems to study lymphocyte physiology as well as the role of oncogene expression in lymphocyte growth deregulation.

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Erratum

Energy conversion catalysis using semiconducting transition metal cluster compounds

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Nature **323**, 431–432

In this letter, the metal atoms in the Fig. 1 legend were incorrectly defined. They should read: ●, Mo; ○, Ru; ⊕, Se.

Stratigraphic record of earthquake incidence

CISNE¹ uses the Lofer cycles of the late Triassic Dachstein Formation (Northern Limestone Alps, Austria and Germany) as an illustration of how stratigraphy may record histories of stick-slip movement and thereby of earthquake incidence along fault zones. As the discoverer of the emergence-submergence oscillations of the Lofer cycles, which I attributed to a sea-level oscillation², I am very interested in Cisne's alternative tectonic way of producing periodic emergence of a platform margin. His model explains why the signs of emergence (microkarst, relict soils) should be restricted to the outer margin of the carbonate platform. But it necessitates some revision of current sedimentological and/or tectonic concepts.

It is necessary first of all to place these Triassic terranes into tectonic context. They are now part of the giant Oberostalpin ('upper Austride') klippe that extends for 450 km, from the Swiss border to Vienna, in itself a patchwork of thrust sheets and klippen of various facies, complicated by high-angle faults, and developed in a complex and controversial history³⁻⁷.

In Triassic time, the terrane was part of a tectonic trough, filled in the north with Triassic platform carbonates, while the southern part was open and accumulated the thin pelagic-hemipelagic Hallstatt beds⁵.

In several places, the toe of the reef complexes interfingers with ammonite-bearing, pink or greenish calcilitites of Hallstatt type. However, the bulk of the Hallstatt deep-water rocks are now found as discreet klippen masses emplaced on top of the platform facies and its deeper Jurassic/early Cretaceous cover.

Cisne's stick-slip fault would have defined the southeastern margin of the platform, separating it from the Hallstatt facies. It would have added to regional subsidence an additional component, either on the platform or on the Hallstatt side. Throwing the Hallstatt side down, relative to the platform, accelerates the subsidence of the platform margin during the 'stick' phase, leading through intertidal into subtidal conditions. The slip phase allows this margin to snap back into emergence, followed by truncation, and resubmergence in the next stick episode. In the alternative case, throwing the platform down slows the platform margin's subsidence during the stick phase, and sedimentation leads to progressive shoaling; then the margin snaps down in the slip phase. Thus, the Hallstatt-down model leads to upward deepening cycles, the platform-down model to upward shoaling cycles. One of the most distinctive

characteristics of the Lofer cycles is their generally upward-deepening character, which has seemed unusual to students of carbonate platforms. If these cycles were caused by stick-slip faulting, then the motion was down on the Hallstatt side.

It is here that difficulties develop. If the Hallstatt basin underwent more subsidence than the platform, it must have subsided by more than the thickness of Norian platform sediments (some 1.5 km) during Norian time. Since the Norian of the Hallstatt facies probably does not exceed a thickness of 150 m, the late Norian calls for a water depth of 1.5–2 km. This depth is about the limit to which pelagic aragonite shells persist on the present sea floor (the aragonite compensation depth). It seems that modern compensation depths go back to the dramatic rise in plankton carbonate productivity in late Jurassic time⁸ earlier carbonate compensation depths lay at far shallower levels. Thus, conventional wisdom assigns the Hallstatt carbonates with their ammonites to water depths of several hundred metres, not the required 2 km.

I can think of three possible ways to escape this dilemma—all of which would require revision of current concepts. One is that the oceans of the Triassic were saturated with calcium carbonate to much greater depths than those of the Jurassic, allowing preservation of aragonitic shells at depths of 1.5–2 km.

A second possibility is that the Hallstatt basin was oceanic, a Triassic antecedent of the Jurassic (Pennine) ocean strip, and that most of the Hallstatt beds were seamount deposits, scraped off during southward subduction of that oceanic basin, and emplaced over the margin of the platform as it began to be subducted in mid-Cretaceous time.

The third possibility is that the basin fronting the Loferite cycles was deep. The Hallstatt beds were deposited in a shallower part of the trough, to the west. The Hallstatt facies was first brought to the longitude of the Lofer cycles by sinistral strike-slip displacements, probably in the mid-Jurassic when the Lamm-Torren zone underwent tens of kilometres of sinistral motion⁵, and were then emplaced by Juvavian (mid-Cretaceous) thrusting.

My preference is for the second of these models, which implies an 'accreted prism' origin for the entire stack of Alpine sediments.

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Primary structure of the histidine-rich protein of *Plasmodium lophurae*

SIR—We wish to address the literature concerning the sequence of the histidine-rich protein (HRP) and the corresponding gene from the avian malaria parasite *Plasmodium lophurae*. Following publication of a DNA sequence derived from a *P. lophurae* genomic clone¹, we were forced to reassess our own data, which differed extensively from the 3' half of the published coding sequence. Our results were derived from the complementary DNA clones² that were used to isolate the genomic clone. A corrigendum to the genomic sequence was published³; in the revised sequence, 120 base pairs (bp) were deleted and 90 bp were inserted. Our analyses⁴ independently suggested that the published genomic sequence contained an insertion and a deletion, each of about 120 bp, together with a region of about 100 bp containing multiple insertions of one or two bp in the genomic sequence. The region of the genomic sequence between the insertion and deletion sites was not revised³. There is a significant difference in the amino acid compositions predicted by the two sequences, the most extreme being a fourfold difference in phenylalanine content.

What then are the most probable structures of the HRP and its gene? Making the assumptions that the published 5' DNA sequence¹ and corrections³ to the numbers of downstream repeats not covered by the cDNA sequence are correct and that the 3' sequence from the cDNA⁴ should take preference, a composite sequence can be constructed. The mature HRP (289 amino acids) predicted by this sequence has a molecular weight of 37,109 and contains 71.3% histidine, 7.2% proline, 7.0% alanine, 6.2% glutamic acid and smaller amounts of eight other amino acids. This composition is consistent with three independent determinations of the composition of purified HRP (cited in ref. 4).

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Quantitative receptor autoradiography

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Quantifying receptor autoradiography is a useful tool, and one becoming increasingly popular, but potential errors exist in the interpretation of results.

RECEPTOR binding studies are powerful weapons in the neurochemist's armamentarium, their present application being predominantly in basic neurochemical research, drug discovery programmes and toxicology studies. Recently their status has been elevated by the increasing sophistication of receptor autoradiography. The predominant advantage of this technique over conventional homogenate-based binding assays is enhanced anatomical resolution combined with an increased sensitivity that is reported to be four to five orders of magnitude greater than "grind and bind" assays¹. The additional advantage of autoradiography is that the distribution of binding sites, revealed as patterns of radioactivity, can be related to detailed structures in the specimen¹.

Here we highlight the dilemma associated with the standardization of receptor autoradiograms, and pinpoint potential errors in the associated calculations.

The technique

Receptor binding to slide-mounted tissue sections is performed essentially in the same way as homogenate-based assays. Sample sections 10 μm thick are mounted onto microscope slides, incubated with radioligand, washed and then dried. Receptor autoradiograms are produced by tightly apposing the tissue to either emulsion-coated coverslips or commercial radioisotopic-sensitive film. Following exposure and development, binding sites are visualized as silver grains in nuclear emulsions or as gray images on autoradiographic film.

Examination of autoradiographic images on emulsion-coated coverslips is facilitated by dark-field microscopy, where the silver grains appear white on a dark background. This technique gives better resolution than film autoradiography because the crystal diameter of the emulsion is smaller than that used to coat films. However, this method is capricious and quantification of silver grains is time-consuming; achieved by literally counting the number of grains per unit area. In contrast, film-based receptor autoradiography is comparatively easy to perform, and allows up to 60 microscope slides to be processed simultaneously. An example of film-based receptor autoradiography is shown in Fig. 1. In film autoradiography the gray levels associated with receptor localization can be readily converted into optical densities using an image analysis

system, to allow quantification. The main disadvantage of film compared to the coverslip technique is that the tissue sections are not attached permanently to the autoradiographic image, making the identification of discretely labelled regions more difficult.

Until recently, a serious disadvantage of autoradiography compared to hom-



Fig. 1 *a*, An autoradiograph demonstrating film-based receptor autoradiography for the binding of cholecystokinin in rat brain.

ogenate-based assays was that it was only quantitative. In 1981, the use of calibrated radioactive standards that were co-exposed with the tissue sections under study was introduced⁴. Two types of standards are currently used. The first is com-

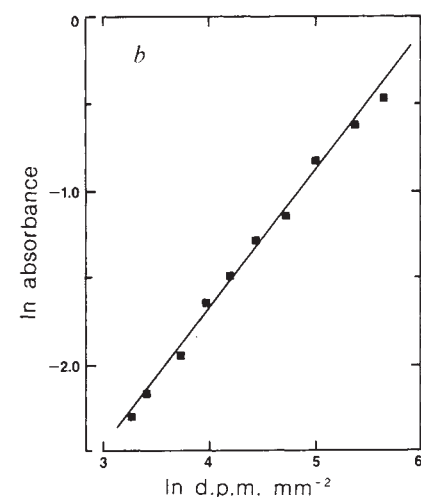
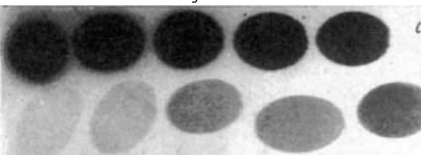


Fig. 2 *a*, Photograph of an autoradiographic image for a series of calibrated [¹²⁵I] brain paste standards. *b*, A natural logarithmic plot of optical density vs. radioactivity per mm² for the series of [¹²⁵I] brain paste standards⁵ in *a*.

mercially available, and consists of increasing quantities of [³H] or [¹⁴C] embedded in blocks of polymethylmethacrylate. Alternatively, standards can be prepared³ in the laboratory by mixing increasing quantities of radionuclide with a tissue paste prepared from the same tissues as those used in the receptor assay. For the assay, a set of calibrated standards (polymer or tissue paste) are cut at the same thickness as tissue sections and are developed alongside the tissue sections. Thus optical densities can be converted into molar quantities of receptor-bound radioligand by comparing the optical density of the tissue sample to the known standard. Figure 2 shows a photograph of a series of [¹²⁵I] brain paste standards exposed to Ultrofilm for 10 days (*a*) together with a natural log plot (*b*) of optical density versus radioactivity contained within each standard section.

Dilemmas of standardization

A major consideration when using [³H] standards is quenching, or the absorption of the low energy β -particles by the medium before they can reach the photographic emulsion. The generation of tissue paste standards is an attempt to circumvent this problem since it is reasoned that the degree of β -particle absorption in tissue paste is the same as that occurring within tissue sections. However, this does not completely overcome the problem, because the degree of quenching of [³H] may differ within tissue types. Absorption in tissues from the central nervous system, for example, has been demonstrated to be different in gray and white matter⁶, and methods for coping with this regional absorption have been discussed⁷. In contrast, self-absorption of β -particles from [¹²⁵I] has been shown in central nervous system tissues to be less significant because [¹²⁵I] emits electrons of higher energies than [³H]. Since [¹²⁵I] polymer-based standards are not yet available, tissue paste standards represent the only way of quantifying receptor autoradiograms in molar terms using [¹²⁵I].

A second problem concerning the standardization of receptor autoradiograms using any radioisotope concerns the frequent, but incorrect interchange of two unrelated terms, "molar quantities of receptor bound ligand per mg of tissue protein" and "molar quantities per mg of standard protein". These are not synonymous, because the protein concentration of tissue paste may not be the same as

that of the tissue under investigation. We therefore recommend adoption of the term dpm mm^{-2} , which can be converted into molar quantities per surface area⁵ since the absolute values for amount of radioactivity, surface area and optical density can be readily obtained from standard sections.

Recently, a further problem concerning standardization has been encountered, during the conversion of dpm mm^{-2} to $\text{fmols bound mm}^{-2}$. When using [³H] labelled material the potential error is negligible because this isotope has a long half-life of 12.35 years. In contrast, the relatively short 60-day half-life of [¹²⁵I] necessitates the regular production of tissue paste standards. In practice, these [¹²⁵I] standards are not prepared at the same time as the binding assay, and are used repeatedly over two half-lives. Calculation errors result when the specific activity of the standard instead of the specific activity of the radiolabel used in the binding assay is used to make the conversion of dpm into fmols . Depending upon the relative age (or decay) of tissue paste standards compared to the radiolabel used in the assay, up to two-fold under- or overestimations of specific binding can occur, and this might have contributed to the considerable differences reported between laboratories of values for fmols of radioligand bound per unit area.

Future potential

Considerable advances have been made in the standardization and quantification of autoradiograms over the past five years. To further improve the technique, the anatomical resolution could be greatly enhanced if the crystal diameter of the emulsion used to make the films was reduced significantly. Theoretically, the sensitivity of the emulsion would also be reduced, but this could be overcome by increasing the time of apposition. The development of three-dimensional image analysis of receptor autoradiograms is inevitable with the rapidly increasing capacities of new computer systems, and this will definitely expand the range of possibilities for the applications of receptor autoradiography.

Martin Hall and Anthony Davenport are at the Parke-Davis Research Unit, Addenbrooke's Hospital Site, Hills Road, Cambridge CB2 2QB, UK, and Colin Clark is at the Pharmaceutical Research Division, Warner Lambert Co., 2800 Plymouth Road, Ann Arbor, Michigan 48105, USA.

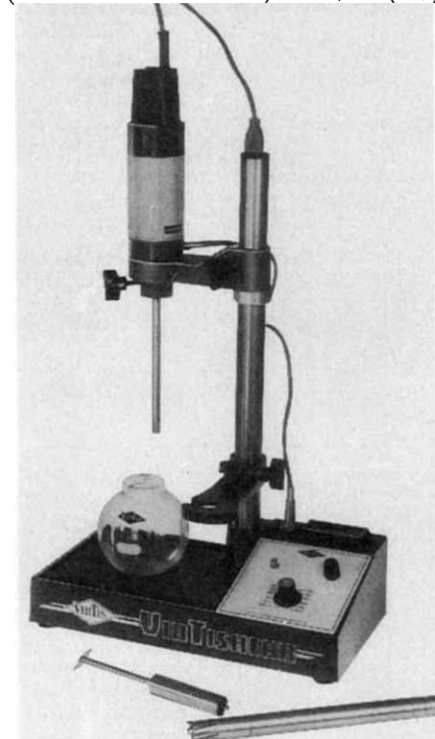
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International exhibits

Highlights from the Salon du Laboratoire and Interchemie '86 in Paris, and from the American Society for Cell Biology meeting in Washington, DC

PROMEGA'S X-Cell electroporation systems are designed for plant cell, mammalian, and bacterial electroporation (Reader Service No. 100). The \$4,950 (US) X-Cell 450 provides a variety of parameter combinations, with a voltage range of 0-450, and a capacitance of 50-1,550 μF , used for mammalian and plant cells, while the \$3,950 (US) X-Cell 2000 provides higher voltages (0-2,000 volts, 5,000 optional) and lower capacitance (14 μF ; suitable for mammalian and bacterial cell electroporation. Both systems employ disposable cuvettes. Promega will be in booths 925 and 927 at the American Society for Cell Biology meeting.

VirTis now offers a **mechanical tissue homogenizer**, designed for the rapid dispersion, emulsification and homogenization of solids, biological tissue and liquids (Reader Service No. 101). The \$895 (US)



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VirTishear can accommodate both open-blade and rotor/stator assemblies (sold separately), and all shafts can be removed for cleaning and autoclaving. The interchangeable shafts allow the operator to process samples from 0.5 ml to 2,000 ml, and standard and specialized accessory glassware is available. For reproducible results, VirTis provides an optional panel-mounted digital speed indicator. Inquire about the VirTishear and other VirTis

products at booths 524 and 526 at the American Society for Cell Biology meeting.

Controlling the temperature of samples immersed in a water bath is often critical for reaction studies. Gallenkamp is distributing a new control head by Haake that sets out to make **temperature regulation** easier (Reader Service No. 102). The Haake F3 control head monitors the temperature of the sample directly from a pt100 sensor positioned in the sample. Temperature controllers are available for a wide range of Haake baths for temperatures between -100 °C and 250 °C, and can be coupled to accessory programmers or computers. Haake's products will be at the Salon du Laboratoire.

Alfa-Laval has been in the fermentation business for a long time, and provides equipment scaled down for the research laboratory, as well as larger setups for industry (Reader Service No. 103). In addition to fermenters, Alfa-Laval has created a **vertical stirrer**, the Vibro-Mixer. The stirrer consists of horizontal plates with vertical cone-shaped nozzles, and as the stirrer moves up and down, liquid is pumped through the cones by the venturi effect. Alfa-Laval says it is ideal for mixing protein solutions that must not be subjected to shear. The laboratory-sized 4-litre E-1 model has a sterile seal, and starts around £500. Interchemie will have an exhibit by Alfa-Laval.

Growth enhancers

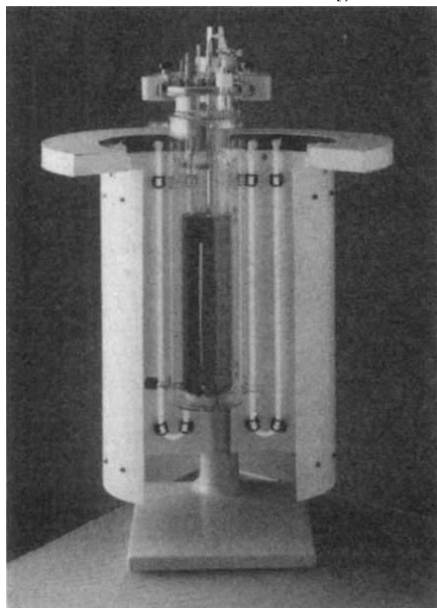
Amgen Biologicals is bringing a host of new **recombinant growth factors** to market (Reader Service No. 104). Platelet derived growth factor, the principal mitogen responsible for the growth of many serum-dependent cell cultures of mesenchymal origin, is prepared from a eukaryotic recombinant DNA host harbouring the *v-sis* gene from simian sarcoma virus. Although it differs from the cellular peptide in four amino acids, it has the same spectrum and potency of biological activity, according to Amgen. Also produced by Amgen is a potent mitogen for vascular endothelial cells and other cells of mesodermal and neuroectodermal origin, basic fibroblast growth factor, for the maintenance of cultures requiring serum-free media preparations. A newly cloned granulocyte colony stimulating factor is offered for research investigating the maturation of neutrophils and the activation of granulocyte-mediated immune

responses, and tumour necrosis factor is available, shown to have tumoricidal activity *in vivo*. The growth factors are sold as 5–100 µg samples ranging in price from \$225 to \$775 (US). Amgen will be exhibiting at the convention of the American Society for Cell Biology in booths 19–21.

For the **radioimmunoassay of growth factors**, Amersham has launched a new line of reagent packs (Reader Service No. 105). The kits for IL-2 and IGF-1 contain one iodinated tracer, matched antisera, a buffer formulated for use in Amersham's Amerlex-M magnetic separation system and a protocol booklet. Ask about this and other Amersham products at its booth at the Salon du Laboratoire and in booth 1018 at the American Society for Cell Biology meeting.

Cell specifics

For the **cultivation of photosynthetic organisms**, LH Fermentation Ltd makes a light bank for the LH500 series of airlift fermenters (Reader Service No. 106). The light bank is mounted on the pillar of the airlift fermenter stand, and fully encircles the fermenter with light. The



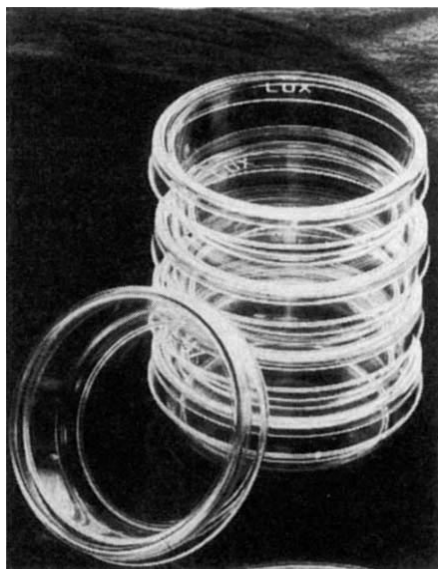
The LH Fermentation light bank sheds light upon photosynthetic cell cultures, stimulating their growth.

£1,329 (UK) light bank consists of two semicircular halves, each containing three U-shaped fluorescent lamps, that open to allow access to the fermentation vessel. The intensity of illumination can be varied to accommodate the needs of different cells. See LH Fermentation's products at the Interchemie Exhibition.

To sterilize complex culture media without autoclaving, Gelman Sciences, Ltd produces a disposable, **ready-to-use capsule filter** (Reader Service No. 107). The presterilized capsule incorporates a

double layer 0.2 µm low-protein binding HT Tuffryn membrane that sterilizes up to 10 litres of serum-free media. The capsules cost £10 each. A selection of Gelman Sciences' other cell culture products will be on display in booth 514 at the American Society for Cell Biology meeting.

The **Permanox tissue culture dishes** developed by Miles Laboratories are formulated to transmit extremely low levels of molecular oxygen, thereby facilitating the anoxic growth of cancer cells for research purposes (Reader Service No. 108). The



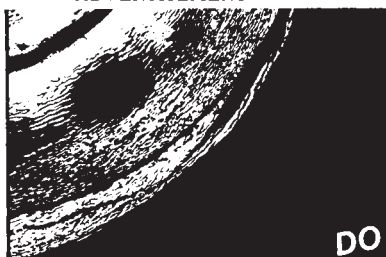
Grow, fix and embed for electron microscopy all in one oxygen-free dish

non-styrene material is also highly resistant to chemicals such as acetone and propylene oxide, so cultures may be grown, fixed and embedded for electron microscopy in the same dish. The Permanox products are a 35 × 10 mm dish, a 60 × 15 mm dish, and a 25 × 76 mm slide. The new 35 × 10 mm dishes are sold for \$189 (US) in lots of 500. Miles Laboratories will be exhibiting at the Salon du Laboratoire, and at booth number 623 at the American Society for Cell Biology convention.

Uniscience, Ltd. is now distributing Wheaton's **roller culture apparatus** (Reader Service No. 109). Wheaton's modular cell production apparatus is designed for the large-scale propagation of monolayer cell cultures, and up to eight decks, holding five bottles each, may be added to the base unit. The £3,600 (UK) roller (including eight decks) features a control unit with a locking shaft, and rotates the bottles at speeds of between 0.125 and 6.9 r.p.m. with an accuracy of ±1%, according to Wheaton. See Wheaton's other wares at their booth at the Salon du Laboratoire, and at booths 610 and 612 at the American Society for Cell Biology meeting.

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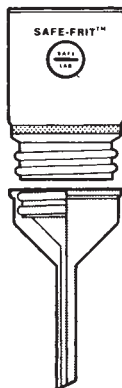
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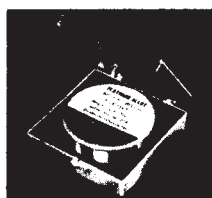
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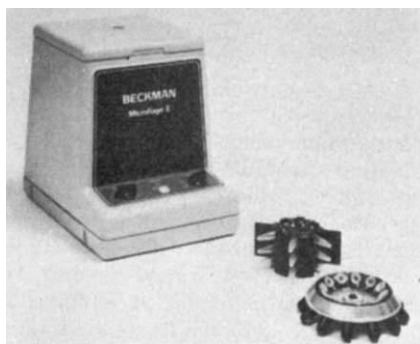
Sample spins

The CT411 thermostated benchtop centrifuge sold by Jouan, Inc. has both a refrigeration and a heating unit, with the temperature range 0-60°C (Reader Service No. 110). Jouan says its \$5,500 (US) cen-

*Spins hot or cold*

trifuge can attain a speed of up to 5,840g and that its digital readout is accurate to 10 r.p.m. Standard features are a safety lid interlock, a continuously variable braking rate and an imbalance detection and shut-down system. Jouan will have a booth at Salon du Laboratoire.

Beckman sells a miniature tabletop centrifuge that it says is ideal for repetitive runs of small biological samples (Reader Service No. 111). The \$845 (US) Micro-

*Just the thing for tiny amounts*

fuge E uses either a horizontal rotor (\$130 US) capable of speeds of up to 14,500 r.p.m. at forces of up to 13,490g, or a fixed angle rotor (\$150 US) that runs up to 15,000 rpm at centrifugal forces of up to 15,850g. It holds from 12 to 48 microtubes, 250 µl to 1.8 ml in size and has a "momentary" button for one-second spins. Beckman will be displaying its centrifuges at the Salon du Laboratoire and at the American Society for Cell Biology meeting.

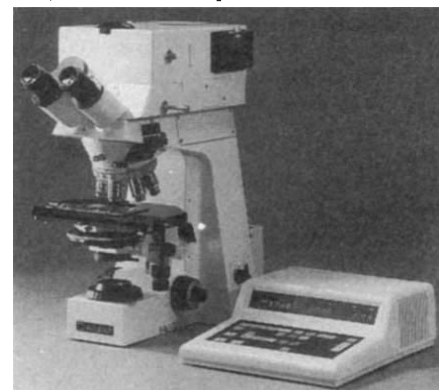
To make sure the right bottle is being used for the right rotor, Nalgene has compiled a *Centrifuge Tube and Bottle Rotor Matching Guide* (Reader Service No. 112). The free 12-page guide contains tables matching the appropriate Nalgene tube or bottle to the rotors of nine different manufacturers. Tips on sterilization,

sealing caps and cap assemblies, and a chemical resistance chart are also included. Nalgene has an exhibit at the American Society for Cell Biology meeting, booth 301, and will also be present at Salon du Laboratoire.

Costar's microcentrifuge filters can filter volumes of 25 µl to 500 µl with a retained volume of less than 5 µl, according to the manufacturer (Reader Service No. 113). The Spin-X filters are available in two pore sizes, 0.22 µm or 0.45 µm, and several membrane types, including an ultra-low binding cellulose acetate. Costar is in booths 54 and 536 at the American Society for Cell Biology meeting.

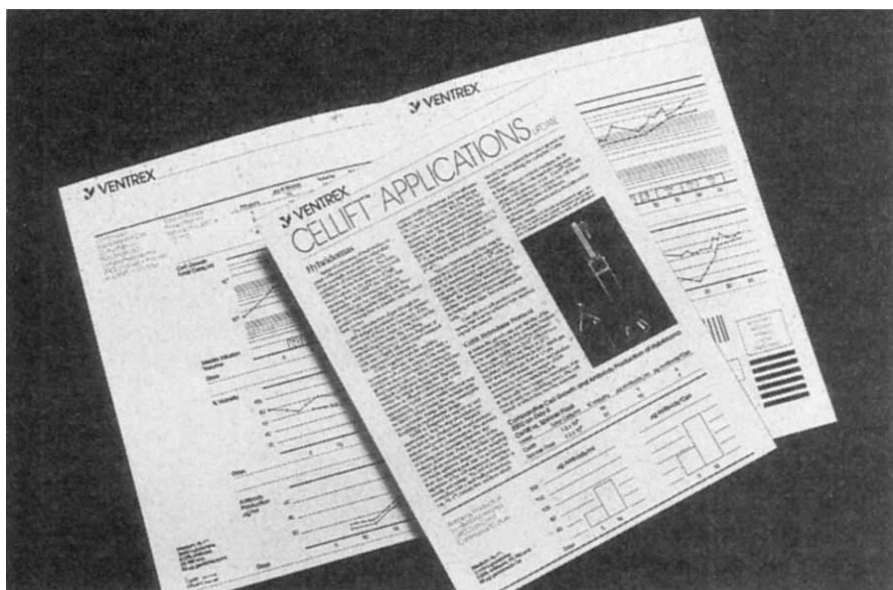
The better to see...

For improved resolution and image quality in photomicroscopy with no sacrifice in field of view, Carl Zeiss, Inc. has developed the Axiophot advanced research microscope (Reader Service No. 114). The Axiophot contains ICS

*Captures important slides on film*

(infinity-colour-corrected system) optics to ensure clear, aberration-free images over a 25 mm field, according to Zeiss, and all components are system-integrated (SI Design). The Axiophot has two 35 mm built-in microprocessor-controlled cameras operated with an external keyboard, and carries a \$32,000 (US) price tag. Carl Zeiss has an exhibit booth at the Salon du Laboratoire and will be in booths 618-626 at the American Society for Cell Biology meeting.

The PL Fluotar series of microscope objectives from Leitz are ideal for fluorescence microscope and interference contrast work, according to the manufacturer (Reader Service No. 115). They offer high near-ultraviolet transmission, minimal autofluorescence and negligible strain, and provide neutral colour reproduction and a field flat to 25 mm. The objectives range in price from £329.50 (UK) for the 6.3× objective, to £348 (UK) for the 25× objective. More information on Leitz's objectives will be available from the Leitz booth at the Salon du Laboratoire, and from booths 1036 and 1038 at the American Society for Cell Biology meeting.



The book with the lowdown on the Ventrex range of airlift fermenters

Lots of literature

A recent release from International Bio-Technologies Ltd, *Cancer Research News*, outlines the role of extracellular matrix (ECM) in the field of cancer research (Reader Service No. 116). The six-page



International Bio-Technologies highlights the latest techniques in culturing cancer cells

brochure aims to introduce International Bio-Technologies' line of ECM-coated labware, but also contains several interesting articles on the culture of cancer cells in general. *Cancer Research News* also offers an introduction to International Bio-Technologies' library of other free brochures, on topics ranging from immunology to neurobiology. International Bio-Technologies will have a display in booth 1035 at the American Society for Cell Biology meeting.

Ventrex Laboratories, Inc. will be on hand in booth 806 at the American Society for Cell Biology meeting, and will be able to provide information about *Cellfit Applications Update* (Reader Service No. 117). This technical publication outlines the procedures for using Ventrex's Cellfit disposable airlift bioreactor. Ventrex says Cellfit's advantage is the provision of a well-mixed culture in the absence of high shearing forces, and is ideal for the culture of a wide range of cells, from mammalian to yeast.

Free custom culture optimization is only one of the services provided by Scott Laboratories (Reader Service No. 118). With the guaranteed purchase of a minimum order of growth media, Scott will work out the optimum growth conditions of a customer's culture, using a client-supplied cell line sample and analysis of spent media. Scott claims a 70 per cent success rate for its service so far, with past results yielding 2–50 fold increases in product. Scott's new catalogue, giving full descriptions of their other services, media, and growth factors, will be on display at booth 330 at the American Society for Cell Biology meeting.

An easy wall chart reference for the preparation of cell culture media is available free from Imperial Laboratories, as a product insert (Reader Service No. 119). It gives the formulations of the most commonly used media, including information on reconstitution, and quantities of sodium bicarbonate and L-glutamine required for various media. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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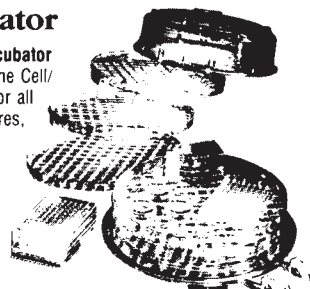
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Reader Service No.7

Strategy for higher education

Richard Pearson

Modest increases in government spending on universities announced recently do nothing to fulfil the urgent need for a long-term strategy for higher education.

It is now 18 months since the government published its consultative Green Paper on the future of higher education. Since then higher education has staggered on, responding to piecemeal evolution of a policy, which until last month has mainly resulted in a tightening of the financial screw. November saw a slight improvement, however, with a small increase in the universities' budgets as part of the government's boost to public spending. Whether this signals a change of policy is not clear and the government's White Paper on the future of higher education still seems many months away. Higher education will remain in a state of crisis and policy decisions by students and institutions will have to be taken on an *ad hoc* basis until the government sets out a long-term strategy.

Although some academics still fear a threat to academic freedom, it is clearly right that the role and running of a higher education system costing the public purse over £3,500 million each year should be a matter for public debate and scrutiny. But it would be wrong to seek change through a series of partial solutions and short-term actions that cause damage in the long term, while providing no strategic direction for the future. Major unresolved issues include those of student numbers, subject balance, student finance, and academic salaries and resources.

In the past the driving force influencing the size of higher education has been student demand, and particularly the wishes of 18-year-olds. But, with a student population of about 500,000, the United Kingdom has low participation rates in higher education by international standards especially by women in technological subjects, mature entrants and ethnic minorities. The proportion of entrants from working class homes, at under 10 per cent, has barely changed for a decade. Participation by these groups needs to be increased, both for social reasons and to broaden the quality and range of experience in the graduate population which is too narrowly based. Such an increase would counteract the reduced demand for places due to the expected fall in the number of 18-year-olds in the period to 1995. Demand for graduates is now at record levels and expected to increase significantly, and the longer term need is for a better educated and more skilled workforce.

These social and economic objectives of higher education need not be incompatible. For example, the labour market too

needs higher education to have a broader base than is provided by simple reliance on vocational subjects, because employers need a broad cross-section of skilled and educated staff. In the vast majority of jobs and careers, indeed there is no close link with degree subject, so that one in three of current vacancies for graduates do not specify a degree subject. This allows flexibility in the provision of courses for the majority of places and responsiveness to student demand and other criteria when planning subject balance. It also allows rebuttal of the complaint that precise manpower planning does not work, because such accuracy is simply not needed.

There is, though, a need to improve the content and quality of many courses to encourage communication skills, team work, numeracy and literacy. In addition a clarification of which are truly vocational courses needs to be undertaken and a better understanding of employers' current and future needs for specialist trained graduates is required.

More students, better salaries to retain and attract good teachers and adequate, up-to-date resources all cost money. They cannot realistically be paid for by savings from a loan scheme to replace student grants. Industrial support by sponsoring students, donating equipment, funding research, paying for visiting lecturers and financing higher academic salaries, all help higher education as well as the recruitment and research of the firms themselves. There are no exact figures on the full extent of direct industrial support, but overall it is probably less than £100 million, or under 3 per cent of the higher education budget. Thus even the expected significant increases over the next decade will not be enough to relieve the burden on the public purse, though it will continue to make a valuable contribution in selected areas. While improvements in efficiency can also undoubtedly be achieved, the necessary improvements in higher education cannot be realized without a reducing budget; public financing has to be increased in real terms, not reduced.

A better way of distributing finite resources may be through the introduction of the two-year general degree, topped up by a two-year specialist degree. The first degree would be available to all; it would offer a broad-based curriculum and would attract a maintenance grant. Student demand could strongly influence subject balance as long as there were some core

general subjects which would compensate for any over specialization in the schools. Such degree courses could offer far more flexible entry qualifications, which would allow more mature students and those from less conventional backgrounds to enter higher education.

There would then be a smaller number of places on second degrees which would be more specialized, focusing on particular disciplines. The balance would be determined more by national or occupational criteria, as already happens in subjects such as medicine and architecture, or by the need for advanced study, as is the case for postgraduate courses in the arts and many of the social sciences. These subjects would attract further grants, scholarships or industrial sponsorship in key areas of the labour market and loans could also be available. A critical factor in their implementation would be that all degree courses should follow this pattern — otherwise the new two-year degree courses would be instantly dubbed second class.

Whatever the strategy for higher education — and the options are diverse — there must be a long-term perspective and an improved planning framework, which would require better information about inputs, processes and outcomes. If market forces are to play a key role then participants have to be better informed: students about job prospects to help in the choice of subject and course, institutions about economic needs to tailor a proportion of the courses accordingly, and government in terms of deciding how much money to spend, and where and how to allocate it. All these groups require a better articulation of the longer-term relationship between economic and industrial needs and the provision of higher education, so that priorities can be considered, and flexibility built into the system to cope with uncertainty. The performance of higher education as a whole also requires regular monitoring and policies must respond to changing economic and social needs.

Whatever the complexion of governments in the next decade there is unlikely to be a significant increase in the funding of higher education. Piecemeal policy adjustments are likely only to reinforce the downwards spiral and inhibit a successful transition into the 1990s. A longer-term strategy is vital. □

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